Trichuris suis ova in the Treatment of Inflammatory Bowel Diseases

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Zürich, 2015

"Why is a raven like a writing desk?"

Lewis Carroll, 1865

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ABBREVIATIONS

5-ASA. 5-aminosalicylic acid
6-MP. 6-mercaptopurine

aaMac. alternatively activated macrophages
Ag. antigen
AIH. autoimmune hepatitis
APC. antigen presenting cells
Asc. Adult *A. suum* extract
AZA. azathioprine

CD. Crohn's disease
CIA. collagen induced arthritis, rheumatoid arthritis
COX. cyclooxigenase
CycA. cyclosporine A

DAMP. danger associated molecular patterns
DC. dendritic cell
D-GalNAc. *N*-Acetylgalactosamine
DSS. dextran sodium sulphate

E/S. excretory/secretory products
EAE. experimental autoimmune encephalomyelitis
GC. goblet cells

i.g. intra gastrically
i.p. intra peritoneum
i.v. intravenous
Ig. immunoglobulin
IBD. inflammatory bowel disease
IEC. intestinal epithelial cells
IL. interleukin
ILC. innate lymphoid cell
KO. Knock out

LPMC. lamina propria mononuclear cells

Mac. macrophage
MDP. muramyl dipeptide
MHC. major histocompatibility complex
MIF. macrophage inhibitory factor
mLN. mesenteric lymph node
MP. methylprednisolone
MR. mannose receptor
MS. multiple sclerosis

NLT. NOD like receptor
NKT. natural killer T cells
OR. odds ratio

p.o. per oral
PAMP. pathogen associated molecular patterns
PC. phosphorylcholine
PRR. Pattern recognition receptors

RA. rematoid arthritis
RORC. retinoid orphan receptor C
STH. soil transmitted helminthiasis

T1D. type 1 dyabetes
Tc. cytotoxic T cell
TGFβ
Th. helper T cell
TJ. tight junctions
TLR. toll like receptor
TNBS. trinitrobenzene sulfonic acid
TSO. *T. suis ova*
UC. ulcerative colitis

SUMMARY

Helminth parasites may be regarded as intestinal symbionts that co-evolved with the immune system and are thereby essential for its proper maturation and functioning. According to the helminth therapy concept, the modulation that helminths exert on the immune system can counteract the dysregulation that occurs in different organic diseases.

Clinical trials in inflammatory bowel diseases (IBD) have been performed mainly with *T. suis* ova (TSO). Despite the first promising open studies that showed a therapeutic effect in both Ulcerative Colitis (UC) and Crohn's disease (CD), larger controlled, randomized multi-zentrische studies failed to show a significant effect of TSO in comparison to placebo in mild to moderate CD patients. The available evidence in support of its efficacy and safety is complicated by the lack of systematic studies in immunosuppressed individuals that constitute the majority of the IBD patients.

Two major obstacles complicate translational research with *T. suis*. First, the parasites fail to colonise the intestine of mouse and rats. Second, in rabbits -where *T. suis* follows the life cycle observed in humans- a suitable IBD model was missing.

To overcome these hurdles, we developed a novel protocol to induce a colitis in rabbits using dextran sodium sulphate (DSS). This model allowed the evaluation of the efficacy and safety of TSO treatment in immunocompetent and immunocompromised hosts.

We show that TSO ameliorate the development of colitis in immunocompetent rabbits, by limiting weight loss and reducing the DSS-induced histopathology. Transcriptome analysis by RNAseq reveals that TSO strongly affect the response of LPMC to the DSS induced injury. In particular, TSO modulates innate inflammatory and cell-adhesion pathways. The weaker modulation exerted on intestinal epithelial cells (IEC) suggests that *T. suis* excretes or secretes molecules that bypass the EC barrier and directly interact with the lamina propria environment.

The preventive immunomodulation is lost in immunosuppressed animals, where TSO treatment exacerbates the colitis. The presence of larvae in the caecum of immunosuppressed rabbits demonstrates a failure in the control of the helminth infection.

Our findings highlight that TSO treatment can be detrimental to immunosuppressed patients as immunosuppression predisposes to adverse effects of helminth therapy.

ZUSAMMENFASSUNG

Die Besiedelung mit Helminthen löst eine Immunreaktion aus, die anti-inflammatorisch auf die überschiessende Immunreaktion während Immunerkrankungen wirken kann. Initiale Studien an Patienten zeigten dass die Verabreichung von Eiern des Schweinepeitschenwurms Trichuris suis (*T. suis* ova, TSO) zu einer deutlichen Verbesserung des Krankheits-Aktivitätsindex bei chronisch entzündlichen Darmerkrankungen führt. Randomisierte, kontrollierte Mulitcenter Studien konnten diesen Effekt jedoch nicht bestätigen.

Genauere Untersuchungen zur Wirkungsweise einer TSO-Therapie konnten bislang aufgrund des Fehlens eines geeigneten Tiermodells nicht durchgeführt werden. Eine TSO-Infektion mit ähnlichem Verlauf wie er für den Menschen beobachtet wird, konnte nur für Kaninchen und Affen gezeigt werden. Die existierenden Kolitis Modelle in Kaninchen waren jedoch für die Untersuchungen zur Wirkungsweise einer TSO-Therapie nicht geeignet. Daher etablierten wir ein Dextran Sodium Sulfat (DSS) Modell einer akuten Kolitis im Kaninchen. Anhand dieses Modells konnten wir die Wirkung von TSO in einem präventiven, therapeutischen Ansatz in immunkompetenten und immunsupprimierten Tieren untersuchen.

Wir konnten zeigen, dass TSO den Kolitis-assoziierten Gewichtsverlust verhindert und die Schwere der Entzündung im Cöcum reduziert. Eine Analyse des Cöcum-Transkriptoms zeigt einen dramatischen Effekt von TSO auf mononukleäre Zellen der Lamina Propria (LPMC). Im Besonderen hemmt TSO angeborene inflammatorische Prozesse ("innate immunity") und Zelladhäsionsprozesse, welche durch DSS induziert sind. Der geringe Effekt auf cäcale Epithelzellen (IEC) weist darauf hin, dass exkretorische oder sekretorische Produkte von *T. suis* die Epithelbarriere überbrücken und die Lamina Propria Umgebung direkt modulieren.

Unsere Versuche weisen jedoch auch nach, dass TSO die akute Kolitis in immunsupprimierten Kaninchen verschlechtert. Die Präsenz von adulten Trichuris im Cöcum deutet darauf hin, dass immunsuppriemierte Kaninchen eine *T. suis* Infektion nicht kontrollieren können. Unsere Erkenntnisse liefern wichtige Hinweise darauf, dass TSO-Behandlungen in immunsupprimierten Individuen zu unerwünschten Nebenwirkungen führen können.

1 INTRODUCTION

1.1 Preface

This thesis investigates the safety and efficacy of a therapy with *T. suis* ova in a rabbit model of inflammatory bowel disease (IBD).

To contextualise our work, I firstly introduce the disease and the various animal models used in translational research. I touch the current available therapies and their limitations as well as the still unclear aetiology of IBD.

The idea that a parasitic pathogen might treat an organic disease is fascinating. In chapter 1.3, I describe the rationale of helminth therapy from an epidemiological and evolutionary perspective. I then review the literature on the therapeutic and preventive use of helminths for organic diseases other than IBD.

In chapter 1.4, I address helminth therapy in IBD. Afterwards, I summarise the experience in IBD models with several helminths species. In the final chapter, I present the particular case of *T. Sui*.

Finally, I end by exploring the clinical studies with *T. suis* and the concerns about its safety and efficacy.

1.2 Inflammatory Bowel Diseases: an Exemplary Post-industrial "Epidemic" Disease

1.2.1 The History of IBD

The term ulcerative colitis (UC) was first used by Wilks in 1859 to describe a case of a non-infectious severe and persistent diarrheal disease[1]. By the start of the 20th century, UC was widely recognized as a disease entity distinct from the intestinal inflammatory pathologies of known aetiology[2]. In 1761, Morgagni, citing the work of his mentor Antonio Maria Valsava-described the case of a 20 year old man who presented abdominal pain, diarrhoea and fever. Following a remission period, the patient developed acute fever, accompanied by stupor and hearing loss and died after two weeks. The Autopsy showed hallmarks that we nowadays can ascribe to Crohn's disease (CD) such as perforations, gangrene, transmural inflammation and ulceration of the terminal ileum and proximal colon[3].

In 1932, Crohn, Ginzburg and Oppenheimer, accurately described cases of chronic terminal ileitis, occurring mainly in young adults, presenting symptoms often observed in ulcerative colitis patients[4]. Although the authors stated that the disease never affected rectum and colon, it was later recognized that the pathology can occur in all regions of the gastrointestinal tract[5]. In 1960, the term Crohn's disease was firstly used to describe a chronic inflammatory bowel disease distinct from ulcerative colitis[6]. Later, the term indeterminate colitis was introduced for the cases where a clear distinction between UC and CD was not achieved[7].

From the initially sparse account, IBD incidence and prevalence experienced a dramatic increase, particularly in industrialized countries. An exemplary case is a retrospective study of IBD epidemiology performed in Rochester, NY: UC was the first disease to emerge in the 20ties (two cases), and CD followed in the 30ties with five cases. The incidence for both diseases rapidly increased and CD soon overtook UC: in the 80ties UC and CD annual incidence respectively reached 23.19 and 39.02 cases/100'000 patients[8]. In Europe and North America the annual incidence currently ranges from 1.5 to 20.3 cases/100'000 patients for UC and 0.7 to 14.4 cases/100'000 patients CD[9]. In developing countries, IBD was practically absent but the incidence is clearly rising[10]; UC annual incidence is still generally greater than that of CD. However, recent studies suggest that, similarly to what is observed in industrialized countries, UC incidence reaches a plateau, while CD incidence continues to increase[11].

1.2.2 Pathophysiology of IBD

Most of the early clinical observations presented above are still valid today. At present, UC is described as a relapsing-remitting chronic inflammatory disease involving a mucosal inflammation without the presence of granulomas. UC involves the rectum and a variable extent of the colon in a progressive and continuous manner (Figure 1.1).

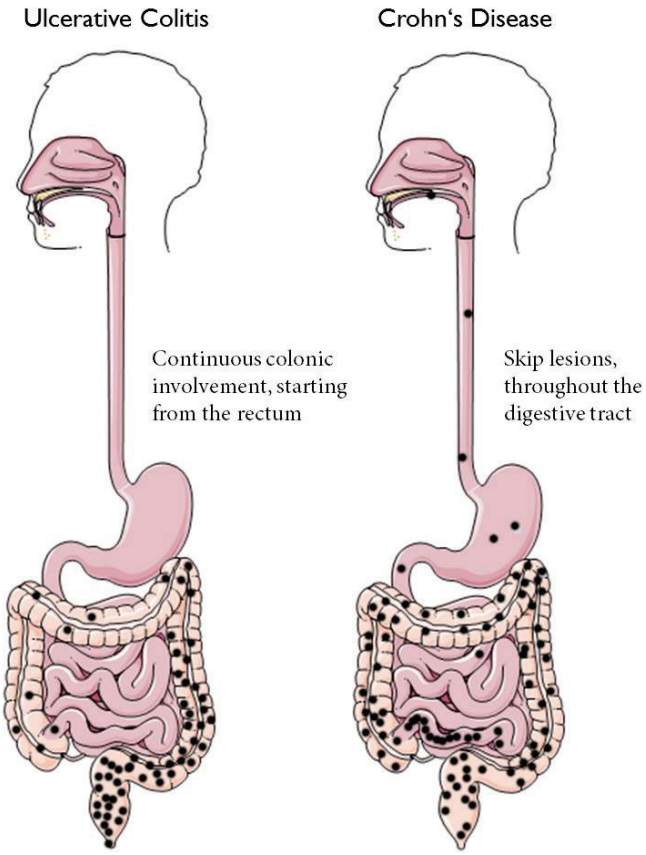

Figure 1.1: Distribution of lesions in ulcerative colitis and Crohn's Disease. Adapted from Kaestner, et al 2009[12].

CD is defined as a chronic and progressive disease involving a nonspecific granulomatous inflammatory process transmural inflammation, strictures, and fistulas.

Table 1.1: Clinical features of UC and CD

Ulcerative Colitis	***Crohn's Disease***
0.5-24.5 cases per 100,000 new persons-years	0.1-16 cases per 100,000 new persons-years
Begin in the rectum with proximal, continuous extension	Any portion of the gastrointestinal tract Segmental involvement
No "skip areas"	Inflammatory disease, strictures, and fistulas.
25% of cases confined to the rectum	35% of cases in the ileum and colon;

75%, proximally and contiguous spread.	32%, solely in the colon; 28%, in the small bowel; 5%, in the gastro duodenal region
Mucosa and the submucosa involved Thickening and dense infiltration of neutrophils, monocytes, macrophages, T cells typically on the mucosal layer	Transmural, all intestinal layers involved Significant thickening Infiltration of macrophages, monocytes and T cells on the sub-mucosal layer
Missing genetic contribution: 84%	Missing genetic contribution: 77%

CD can affect any region of the gastrointestinal tract in a segmented manner, from the mouth to the rectum (Figure 1.1). UC and CD patients suffer from severe diarrhoea, bleeding, abdominal pain, as well as fluid and electrolyte loss. UC and CD also share many extra intestinal manifestations, such as fever, sweats, malaise, and arthralgia.

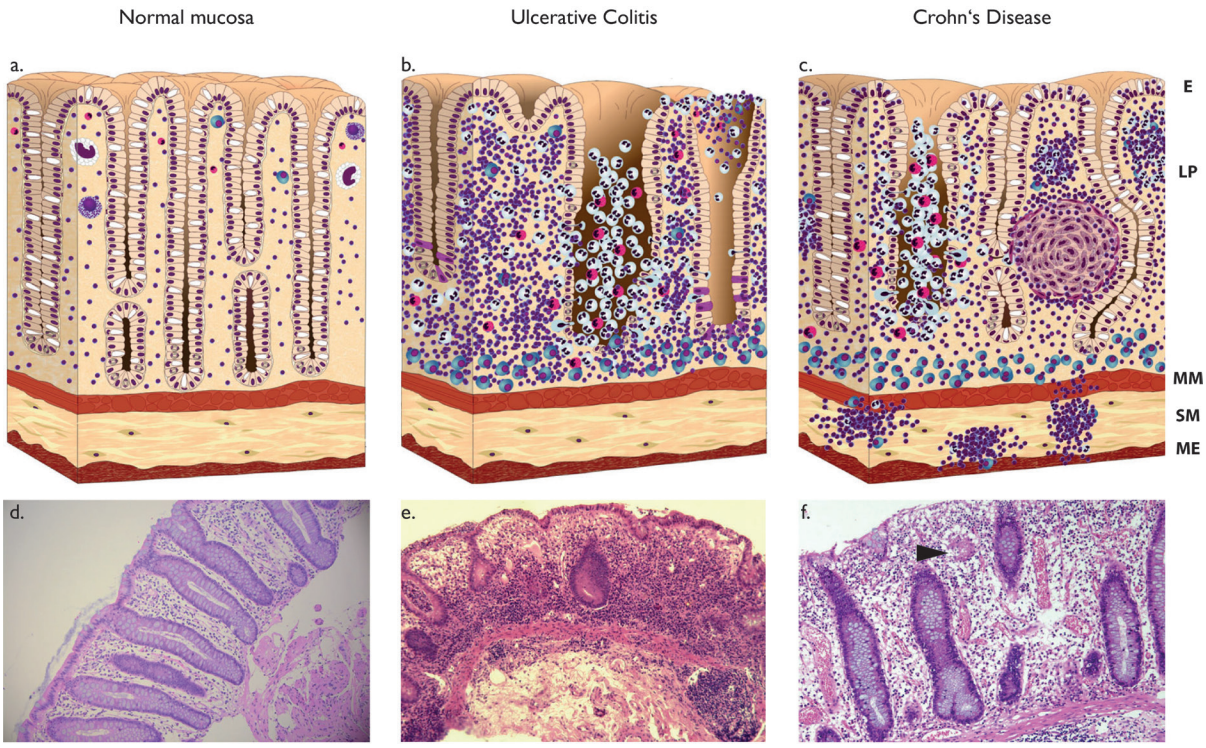

Figure 1.2: Comparison of the microscopic features of normal, UC, and CD mucosa. Active ulcerative colitis shows surface erosion, mucin depletion, crypt abscess, and crypt destruction (b, e). E: epithelium, LP: lamina propria, MM: muscularis mucosa, SM: submucosa, ME: muscularis externa. CD shows a micro granuloma (arrowhead), patchy colitis with irregular villi (c, f). a-c: schematic view, d-f: microscopic features, HE staining. Pictures from Geboes, 2014[14], Van Eyken 2014[15].

The discrimination between the two forms of IBD is especially difficult when the pathology is localized in the colonic region. When it is not possible to reach a definitive diagnosis, the pathology is referred to as IBD unclassified, these cases represent 5-30% of all IBD[13]. An analysis of the histological features of intestinal biopsies is crucial for an IBD diagnosis (Figure 1.2).

Histology also allows an assessment of the disease activity and an early detection of malignancy.

Within the continuous lesion of UC patients, the crypts are scarce and distorted whereas the villi are shorter and stunted (Table 1.2)[15]. Immune cells infiltrate into the deeper level of the lamina propria and plasma cells accumulate in the inflamed mucosa.

The lesions observed in CD are focal and scattered among the normal mucosa. The irregular mucosal morphology is characterised by a reduced density of the crypts that are shorter and dilated. Neutrophils and plasma cells infiltrate the lamina propria and basal lymphoid aggregates form in the susbmucosal layer. Often, monocyte/macrophage cells aggregate with other inflammatory cells in the mucosa to form a granuloma[15].

Table 1.2: Microscopic features of UC and CD

UC	*CD*
Morphology	
Severe crypt architectural distortion	Mucosal surface, normal, irregular, villous
Severe widespread decreased crypt density	Crypt atrophy (shortened, widely spaced crypts)
Stunted villous surface	Distorted, dilated, branching crypts
Inflammation	
Heavy diffuse trans mucosal lamina propria cell increase	Basal plasmacytosis, increase in cells in basal third of lamina propria
Diffuse basal plasmacytosis	Increased lamina propria cellularity (round cells and neutrophils)
Neutrophils infiltration	Basal lymphoid aggregates
Miscellaneous	
Increased intensity of the alterations towards the distal colon	Epithelioid granuloma
Severe mucin depletion	Basal giant cells
Paneth-cell metaplasia distal to the hepatic flexure	Excess Mac and DCs in lamina propria

1.2.3 Aetiology of UC and CD

The heritability of IBD is undeniable but limited: 6-15% of the UC and CD patients have a first degree relative with IBD. Further, in comparison to dyzygotic twins, monozygotic twins have a

higher concordance rate and also experience a similar disease phenotype. In the past decades, linkage studies and genome wide association studies (GWAS) later, allowed the identification of 163 risk loci that are mainly involved in both UC and CD (Figure 1.3). Interestingly, 40% of the IBD risk loci (66 loci) are also found among the 154 loci that are associated with other immune-mediated diseases (Table 1.3)[16].

Table 1.3: IBD risk loci associated with other organic diseases.

Disease	***# common loci***	***Fold-enrichment***	***Enrichment OR***	***P-value***
Psoriasis	14	13.5	14.71	$4.15 \cdot 10^{-12}$
Atopic dermatitis	3	12.1	12.32	$2.05 \cdot 10^{-3}$
Rheumatoid arthritis	12	10.92	11.74	$1.64E \cdot 10^{-9}$
Celiac disease	16	10.57	11.64	$4.56 \cdot 10^{-12}$
Type 1 diabetes	20	9.99	11.28	$2.35 \cdot 10^{-14}$
Multiple sclerosis	17	8.19	9.06	$5.11 \cdot 10^{-11}$
Asthma	7	7.61	7.91	$4.90 \cdot 10^{-5}$

OR: odd ratios; CI: confidence interval (adapted from: Jostins, 2012[16]).

The discovered loci only account for a limited portion of disease variance (the amount of disease that can be explained by these loci): 7.5% in UC and 13.6% in CD[16].

This "missing heritability", arises from a combination of host-intrinsic factors, such as genetic and epigenetic predisposition, immune system defects and environmental factors.

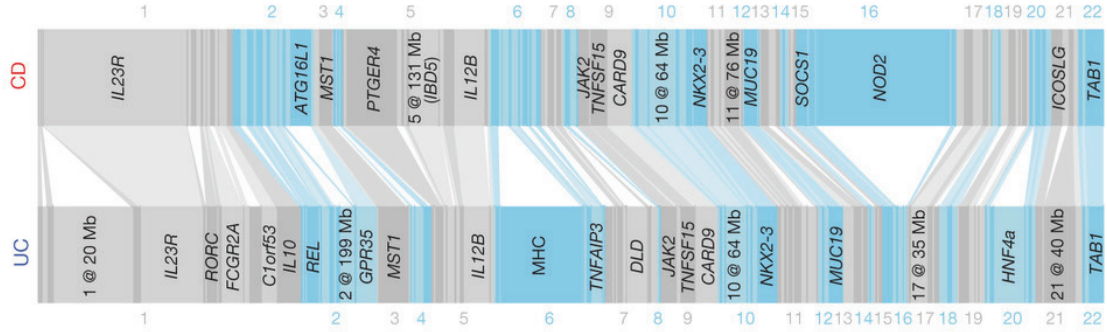

Figure 1.3: Variance explained by the 163 IBD loci. 110/163 loci are associated with both UC and CD, 23 are UC-specific and 30 are CD specific. Each bar, ordered by genomic position, represents a locus and its width is proportional to the variance it explains in CD and UC. Bars are connected together if they are associated with both phenotypes. Labelled loci explain more than 1% of the total variance. Labels are either the best-supported candidate gene or the chromosome position of the locus. From: Jostins, 2012

The complex aetiology also explains the evolving geografic distribution of IBD: the parallel between industrialisation processes and increasing incidence supports the importance of environmental factors. Among the various proposed risk factor, that range from sun-exposure to cornflakes consumption, only few have shown a strong association (Table 1.4)[17; 18;19].

Table 1.4: Selected environmental factors and risk for UC and CD

	Ulcerative colitis		*Crohn's disease*	
Risk factor	West [17; 18]	Asia + Aus[19]	West [17; 18]	Asia + Aus[19]
Smoking				
Current smoker	-		+	+ (Aus)
Ex-smoker	+	+	+	null
Never a smoker	+		-	
Surgery/Medications				
Appendectomy	-		null	
Oral contraceptive pills	+		+	
Antibiotics	?+	-	?+	-
NSAID	+		+	
Diet				
Sugars and fats	?+		?+	
Fibres, fruits, vegetables	?-	- (muesli)	?-	null (muesli)
Coffee (daily)		-		null
Tea (daily)		-		-
Juice		null		-
Breastfeeding	?-	-	?-	-
Hygiene standards				
Sibship	?-		?-	
Urban environment	?+		?+	
Farm in childhood	?-		?-	
Pets		- (Fish)		- (Dog)
In-house water tap/ Hot water tap		-		null
Infections/vaccinations				
M, avium	?null		+	
Pertussis vaccination		-		null
BCG vaccination			+ [20]	+
Dysbiosis	?+[21]		+	
Helicobacter pylori	-		-	
Helminths	-		-	

NSAID: Nonsteroidal anti-inflammatory drugs; *M. avium*: *Mycobacterium avium paratuberculosis* -: clear negative correlation; + clear positive correlation; ?-: weak negative correlation; ?+: weak positive correlation; null: no correlation. West: data for the environmental risk factors in western nations are derived from Molodecky, 2010[17]; Frolkis, 2014[18] and Baron, 2005 [20]; Hold, 2014[21]. Asia + Aus: data for Asian countries and Australia are derived from Ng, 2014[19].

Several proposed factors, such as exposure to infections, antibiotic use, breastfeeding and sibship support the idea of a correlation between hygiene standards and IBD development [17; 18;20;21;19].

1.2.4 Disrupted Intestinal Homeostasis in IBD

A delicate interaction between the epithelial barrier, the immune system and the microbial community governs intestinal homeostasis. Alternations of this balance, caused by a combination of host intrinsic and extrinsic factors, are considered responsible for the development of IBD (Figure 1.4).

1.2.4.1 Intestinal Barrier Function

The intestinal lumen is covered by a network of large highly glycosylated gel-forming mucins that are produced by specialized cells of the intestinal epithelium called goblet cells.

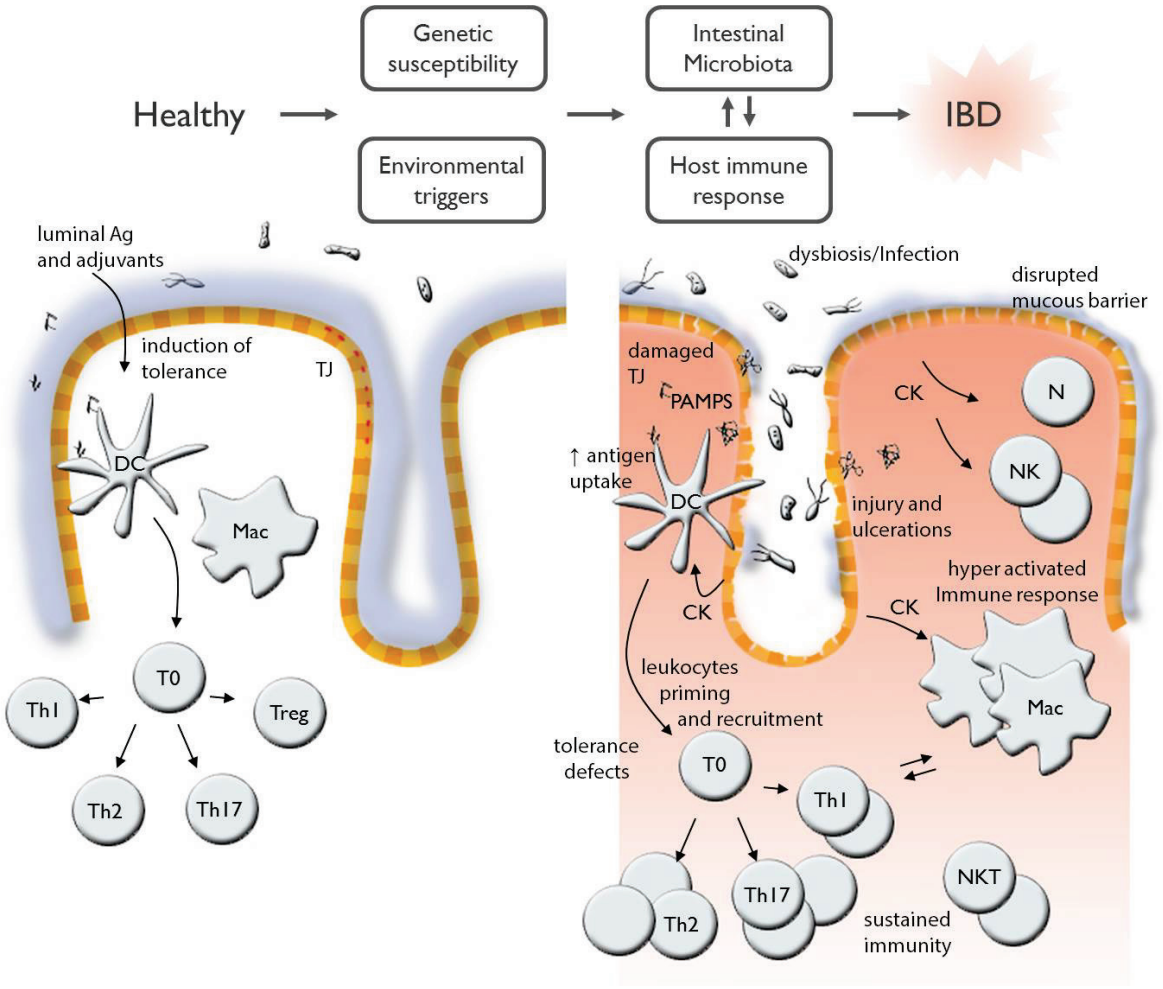

Figure 1.4: Model of the key factors contributing to the disruption of intestinal homeostasis in IBD. Susceptible hosts present underlying genetic defects that affect barrier function, bacterial control and tolerance induction. Environmental triggers contribute to the initiation of aberrant responses toward translocated bacteria and luminal contents that result in progressive damage to the host's intestinal mucosa. The immune response cannot be controlled adequately due immune tolerance defects. Defects in the adaptive response contribute to a chronic immune response towards the microbiota and the host tissues themselves. CK: cytokines, DC: dendritic cells, Mac: macrophages, N: neutrophils, NK: natural killer cells, NKT: natural killer T cells, PAMPs: pathogen associated molecular patterns, T0: naïve T cells, Th: T helper cells, TJ: tight junctions, Treg: regulatory T cells.

The mucous network covers the intestinal epithelium, which consists of a single layer of intestinal epithelial cells (IEC) bound together by a web of tight junctions (TJs). TJs are important regulators of barrier function and paracellular permeability.

In IBD, a dysfunction of these two essential components leads increased barrier permeability. In both UC and CD, an aberrant expression and redistribution of the TJ-forming claudins combined with an increased epithelial apoptosis enhance the barrier dysfunction. In CD patients, GC hyper-proliferate causing a thickening of the mucous layer. Despite being thicker, the mucin network of CD patients shows dramatic changes in the posttranslational glycosylation that compromise its function[27].

Similarly, the mucins' glycosylation pattern observed in UC patients is altered and the degree of distortion correlates with disease activity. In contrast to the hyper proliferation observed in CD patients, GCs of UC patients are depleted and their mucous layer is thinner[28]. Altered TJ structure contributes further to the impaired epithelial barrier function in UC[29]. Different GWAS studies highlighted the link between single nucleotide polymorphisms (SNPs) in TJ-associated genes and the development of IBD. One example is the transcription factor HNF4A that regulates the expression of cell-cell junction components including, adherens junctions, tight junctions and desmosomes[30].

A further contribution to the mucosal damage is caused by the increase matrix metalloproteinases (MMP) produced by fibroblasts, neutrophils and macrophages in response to pro-inflammatory cytokines[31].

1.2.4.2 Innate Immunity

In a genetically susceptible individual, defects in the intestinal barrier function and in the immune response, led to aberrant reactions to luminal antigens and the intestinal microbiota[32]. The innate immune system recognizes microbes and luminal compounds through conserved pattern recognition receptors (PRRs) that allow a rapid and stereotypical response to pathogen associated molecular patterns (PAMPs) and damage associated molecular patters (DAMPs).

PRRs include the membrane bound toll-like receptors (TLRs), C-type lectin Receptors (CLRs) and the cytosolic RNA helicases, DNA sensors and nucleotide-binding oligomerisation domain (Nod)-like receptors (NLR). Intestinal PRR are usually hypo responsive and compartmentalized to ensure that a response occurs only when a pathogen invades the mucosa or penetrate into a cell. Several PRRs have been associated with IBD, highlighting their role in the control of intestinal microbiota and the maintenance of gut homeostasis. For example, UC and CD patients have increased levels of TLR2 and TLR4[33] and CD patients have an increased sensitivity to flagellin, recognized by TLR5[34]. TLR5 loss on IECs leads to an increase in flagellated bacteria, increased colitis susceptibility and metabolic syndrome[35].

The two NLRs NOD1 and NOD2 mediate the response to different peptidoglycan moieties: NOD1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and recognizes muramyl dipeptide (MDP). Mutations in the *NOD2* gene were the first identified susceptibility loci associated with CD, whereas they were later shown to confer protection from UC[36]. The *NOD2* polymorphisms observed in CD cause a reduced NF-κB activation, yet CD patients have an increased NF-κB activation. *Nod2*-KO mice present a defective production of mucus, cryptidins and defensins, and are thus more susceptible to several pathogens. When the *Nod2* gene in mouse is replaced with one of the CD-associated variants, macrophages respond aberrantly to MDP by producing high amounts of IL-1β and exacerbating inflammation[37]. NOD2 also acts in DCs by priming a Th17 polarising phenotype through the release of IL-23 and IL-1[38].

Both macrophages and DC numbers are increased in the lamina propria of IBD patients and their aberrant activation contributes to the induction of a pathologic adaptive response[39].

1.2.4.3 Adaptive Immunity

Early studies suggest that UC and CD patients have an increased clonal expansion of $CD4^+$ and $CD8^+$ T cells in the intestine[40]. The spontaneous colitis observed in C3H/HeJBir mice, is mediated by $CD4^+$ T cells that react to intestinal microbiota antigens[41].

The number of Th1 and Th17 cells is strongly increased in the intestine of CD patients[42]. Th17 cells are in devoted to the defence against intracellular pathogens and are associated with intestinal inflammation and mucosal damage. The levels of Th17 cells and the related cytokines correlate with the disease activity in IBD patients[42]. In the mouse, TGFβ and IL-6 initiate the polarisation of Th17 cells, whereas IL-23 is required for the full effector function[42]. *Il-23r* is a CD susceptibility gene and the associated polymorphisms leads to the induction of a defective Th17 cell effector function[43].

In the T cell transfer model, expression of the gut homing α4β7 expression and intestinal antigen specificity are required for $CD4^+$ T cells colitogenicity. In the same model, colitogenicity is enhanced by IL-23 responsive γδT cells that produce IL-17[44].

Ulcerative colitis patients have a different T-cell profile, characterized by an atypical type 2 response that lacks a clear association with IL-4[45]. This response may be mediated by IL-5 and IL-13 producing natural killer T cells (NKT)[46]. Additionally, imbalances of the homeostatic Treg functions contribute to the abnormal immunity observed in IBD. The differentiation in Treg and Th17 cells is in equilibrium that, when disrupted toward the latter, contributes to intestinal inflammation[47].

1.2.5 IBD and Intestinal Microbiota

The human intestine is home of a complex community of bacteria, viruses and microbial eukaryotes that are referred to as microbiota. The number of bacteria, up to 100 trillion, is 10 times bigger than

the number of cells in the human body. The intestinal bacteria genome contains 3.3 million microbial genes and is thus 150-fold bigger than the human genome[48]. In a healthy individual, 76% of the intestinal bacteria belong to the phylum Firmicutes, 16.4% to Bacteroidetes and the remaining 7% to Proteobacteria, Actinobacteria, Fusobacteria or Verrucomicroba[49].

Recent finding demonstrate a clear segregation between the microbiota communities in the lumen and in the mucous layer. It is important to consider this difference when interpreting microbiota data, since several human studies rely solely on faecal samples[50]. How the different communities affect intestinal homeostasis remains unclear. Evidence suggests that the larger luminal community exerts its influence through co-metabolism or metabolic exchange whereas the mucosal microbiota interact directly with the host epithelium and immune system[51].

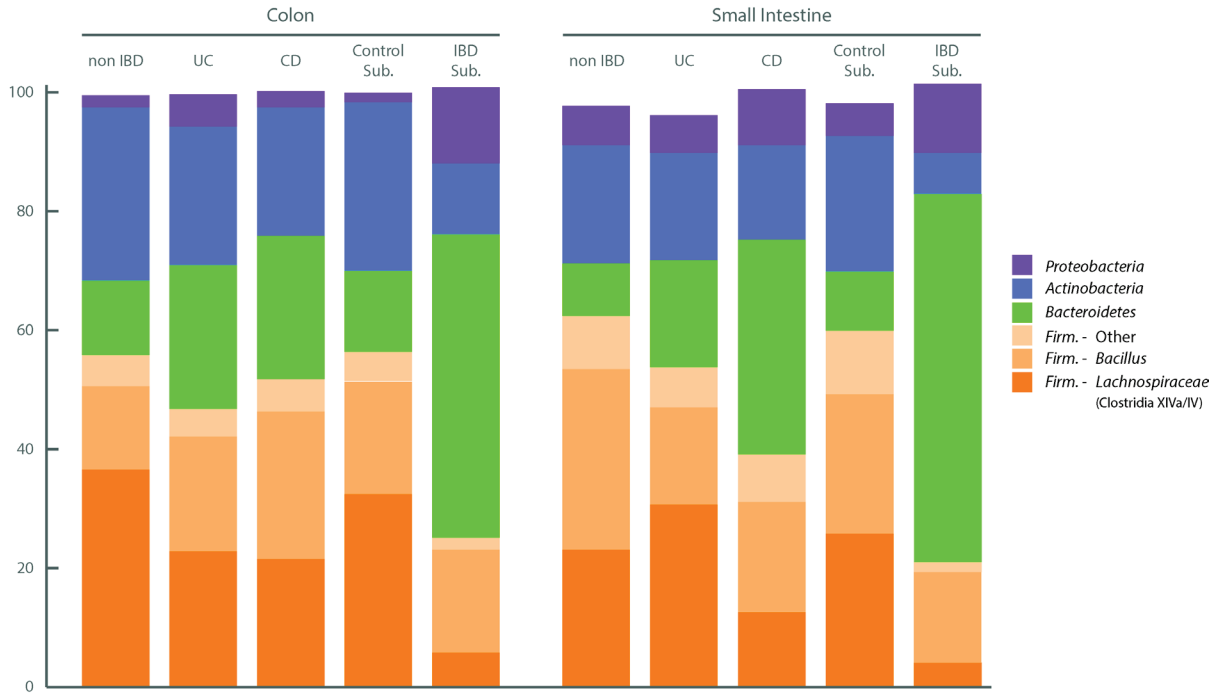

Figure 1.5: Microbiota composition at the Phylum level in the colon and small intestine of IBD patients (UC and CD) and healthy controls. Bars represent percentages of cloned sequences in all samples of a particular category (cfr. legend, Firmicutes are subdivided into Bacillus, Lachnospiraceae, and other groups, Phyla of lower abundance were omitted). Hierarchical clustering of samples on the basis of their first two principal component scores separated the samples into two primary clusters: Control subset (includes most of the non-IBD control samples as well as ca. 2/3 of CD and ca. 3/4 of UC samples) and IBD subset (dominated by CD and UC samples, 39/40 samples). From Frank, 2007[52].

Antibiotics administration during childhood is thought to interfere with the development of tolerance towards commensals and is a known risk factor for both UC and CD[18]. Several pieces of evidence support the role of microbiota in IBD pathogenesis. An alteration of the luminal microbiota composition (termed dysbiosis) is often encountered in IBD patients. The changes observed in IBD

patients are diverse, but they mostly include a reduction in species diversity. A reduction in the number of Firmicutes and Bacteroidetes is frequently observed[52].

Of note, differences also occur among the microbiota associated with inflamed and non-inflamed sites of the same CD patient[49,53,54]. In CD patients, *NOD2* and *ATG16L1* risk alleles are associated with shifts in microbial compositions in particular among Clostridia species[55].

Obviously, these changes might be either a cause or a consequence of the intestinal pathology. Experiments in germ-free mice evidence the crucial role of intestinal bacteria in the maturation of the intestinal immune system. Germ-free mice suffer from an exacerbated colitis upon DSS treatment whereas *Il-10 KO* germ-free mice appear protected from the development of a spontaneous colitis[56]. Microbiota are essential for the proper formation of the intestinal mucosa and GALT (gut associated lymphoid tissues)[57] and for the establishment of oral tolerance[58].

Further, microbiota stimulate the secretion of mucins by goblet cells and the thin mucus layer observed in germ-free mice can be restored to normal levels through exposure to LPS or peptidoglycans (PGN)[59]. Microbiota also influence the adaptive T cell populations both indirectly via modulation of antigen presenting cells (APC) cells and directly via PRR signalling[60]. Another adaptive cell population, NKT, appears to be controlled by neonatal microbial exposure. In the oxazolone colitis model, the pathogenic recruitment of NKT cells observed in germ-free mice is prevented by early colonisation, suggesting a role of this cross talk in the maintenance of mucosal homeostasis[61].

1.2.6 Current Therapies

The complex and unclear aetiology of IBD implies that the therapy is symptomatic and non-curative. The goal of therapy in IBD is at first achieving a response or remission and subsequently maintaining the response or remission[22]. It is important, to carefully evaluate the benefits and side effects of every intervention, with thoughtful consideration of the long-term prospective[22]. The therapy is best tailored by considering both patient factors, such as lifestyle, family history and susceptibility as well as disease factors, such as classification, prior surgery, prognostic factory and disease activity. Further, medication related factors should be considered, for example by assessing the balance between efficacy and safety. The response to previous therapy should also be evaluated and an optimisation effort for the latter should be considered before testing a new one[22,23]. Table 1.5 summarizes the medications commonly used for the therapy of UC and CD.

The anti-inflammatory 5-aminosalicylic acid (5-ASA) exerts a local, topical therapeutic effect on the mucosa. 5-ASA is widely used for the induction and maintenance of remission in UC. The exact therapeutic mechanisms of amino salicylates is unclear; among the recognized properties 5-ASA inhibits intestinal macrophage chemotaxis, reduces antibodies secretions, attenuates the release of

pro inflammatory cytokines and inhibit the arachidonate lipoxygenase and cyclooxygenase (COX) pathways. *Corticosteroids* are widely used for the achievement of remission in both UC and CD. They reduce NF-κB activation and down regulate pro-inflammatory cytokines, mainly through activation of the corticosteroid receptor. Conventional corticosteroids cause a wide range of adverse events, thereby alternative formulations (such as the synthetic prednisolone analogue budesonide) aim to reduce the systemic absorption and increase the therapeutic availability of the drug. Still, classical corticosteroid adverse events might occur especially following a prolonged use.

Table 1.5: Common therapies in UC and CD

Medication	***Indication for UC***	***Indication for CD***	***Side effects***
Anti-inflammatory			
5-ASA (oral, rectal)	induction/maintenance	colonic (mild): induction/maintenance	Intestinal nephritis (rare), diarrhoea
Corticosteroids (oral, i.v., rectal)	induction	induction	Acne, moon face, truncal obesity, osteoporosis, osteonecrosis, diabetes, hypertension, cataract, infections
Immunosuppressive			
6-MP/AZA	corticosteroid withdrawal, maintenance	corticosteroid withdrawal, maintenance	Pancreatitis, fever, infection, leukopenia, hepatotoxicity, lymphoma
Calcineurin inhibitors	corticosteroid refractory		Hypertension, nephrotoxicity, neurotoxicity
Methotrexate	under investigation	induction/maintenance	Nausea, fatigue, hepatotoxicity, pneumonitis
Biologics			
anti-TNF	induction/maintenance	induction/maintenance	Infusion/Injection site reaction, demyelination, infection, heart failure, lymphoma
anti-integrin	anti-TNF refractory; induction/maintenance	anti-TNF refractory; induction/maintenance	Joint pain, fever, PML (Natalizumab, rare)
Antibiotics			
Metronidazole		perianal and colonic disease	Neuropathy, antabuse effect
Ciprofloxacin		perianal and colonic disease	Arthropaty, tendon injury, sun sensitivity

PML: multifocal leukoencephalopathy. Adapted from: ACP, 2012[25].

Azathioprine (AZA) and its metabolite *6-Mercaptopurine* (6-MP) can be administered to UC patients as an alternative to 5-ASA and/or in combination with steroids[24]. They are also used as adjunctive

therapy or as steroid-alternative in patients with active CD and for the remission maintenance in patients with extensive disease. AZA and 6-MP inhibit mitosis and cellular metabolism by antagonising purine metabolism and inhibiting the synthesis of DNA, RNA, and proteins.

The calcineurin inhibitor *Cyclosporine A* (CycA) is a cyclic polypeptide that forms a complex with cytosolic cyclophilins that binds and inhibits calcineurin, thus preventing the dephosphorylation and nuclear translocation of the IL-2 inducing factor NF-AT. This mechanism mainly suppresses cell-mediated immune responses and is used for the treatment of severe UC refractory to corticosteroids. *Methotrexate* is used in active or relapsing CD patients refractory or intolerant to azathioprine or anti-TNF agents although the mechanisms of its anti-inflammatory effect are unknown.

All currently used *anti-TNF antibodies*, such as infliximab and adalimumab, have similar efficacy and similar adverse effects. In UC they are used to induce clinical remission and mucosal healing. In addition, they are used in relapsing CD and for maintenance of remission CD. The *Anti-integrin inhibitors* are an emerging class of biologics that target cell adhesion molecules and their integrin ligands on leukocytes, thus reducing their migration into the site of inflammation. For example, the monoclonal antibody Vedolizumab targets the gut-specific interaction between the integrin α4β7 and MAdCAM-1, specifically blocking T cell homing in the gut[26].

Finally, broad-spectrum antibiotics such as *Metronidazole* and *Ciprofloxacin* show modest benefits for the therapy of colonic CD, either alone or in combination with other drugs.

1.3 Helminth Therapy

1.3.1 "Old Friends" Hypothesis

In the early years of the 19th century, technological innovation and economic growth led to a substantial improvement of the health system, diminishing the exposure to infectious diseases, reducing mortality and increasing the average life span of the population especially in the urban areas. At the same time, industrialising countries experienced a constant rise in the incidence of immune related disorders[62]. Nowadays, a similar phenomenon is occurring in developing countries, and the correlation between increasing hygiene and the development of immune related disorders appears more and more clear.

In 2004, Rook proposed and updated version of the "hygiene hypothesis" that focused on the diminished interaction between the immune system and the environmental microbiota[63, 64].

Partial deprivation of microbial stimuli that were previously abundant shaped the evolution of the human immune system. The immune system could not deal with the "old friends" responsible for these stimuli as it did with usual pathogens leading to the evolution of alternative response mechanisms (Figure 1.6).

Several promising "old friends" candidates have been suggested. On the one hand, commensal organisms transmitted via the orofecal route are harmless, perform essential physiological roles for the host, and thus need to be tolerated. On the other hand, pathogens causing chronic carrier states or non-fatal subclinical diseases also need a customized response[63].

The evolution different subsets of T helper cells, such as Th2/Tregs impedes the complete removal of the chronic pathogens, avoids a destructive inflammatory response and ensures a constant antigen source and immune stimulus, resulting in striking immunity to re-infection[65].

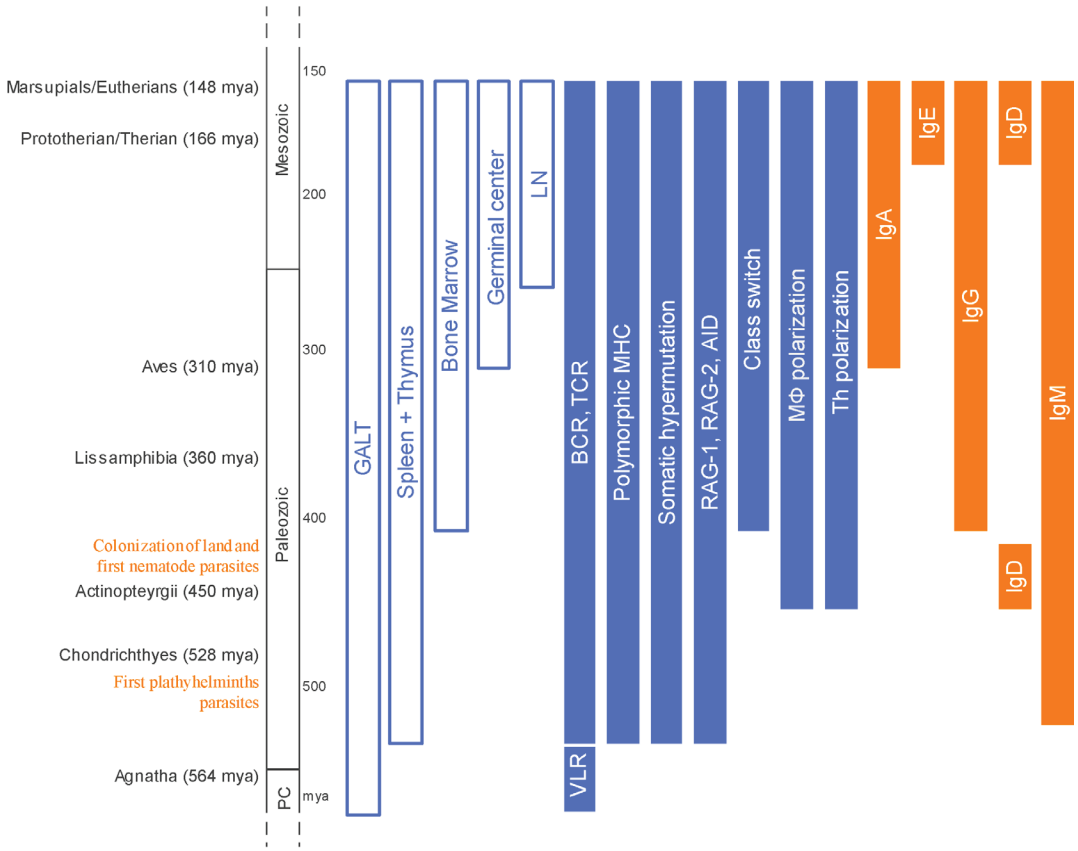

Figure 1.6: Milestones of the immune system evolution in vertebrates occurred in presence helminth parasites. Plathyelminthic parasites colonized the early jaw fishes (Chondrichthyes) about 400 mya. Chondrichthyes were the first animals to develop BCR and TCR recombination (following the acquisition of the RAG gene) and with it a rudimental adaptive response. Nematode parasites colonized terrestrial vertebrates at different time points during the Palaeozoic (from 400 mya), when a fine tuning of the adaptive immune response such as polarization of the T helper cell response and immunoglobulins (Ig) class switch arose. BCR: B cell receptor, GALT: gut associated lymphoid tissues, LN: lymph nodes, PC: Precambrian, TCR: T cell receptor. (adapted from Rook, 2010[69]; Litman, 2005[70]).

A further millstone of the immune system evolution is the development of different Macrophage phenotypes, that are often encountered upon chronic infections[66,67]. Cytokines such as IL-4, IL-13 and IL-21 drive the polarization of alternatively activated Macrophages (aaMacs) that secrete the anti-

inflammatory IL-10 and TGF-β, and contribute to extracellular matrix repair, wound healing and fibrosis[68].

Large parasitic worms (helminths) are a good example of pathogens that need a tailored immune response since a classical inflammatory response would merely cause undesirable immunopathology without antagonizing the parasite[69]. In order ensure the propagation of the species, helminths needed (and need) to escape the host defences and establish a prolonged colonization.

Two groups of helminths evolved zoo-parasitism: Platyhelminthes (flatworms) parasitism probably arose from a common parasitic ancestor in the first jaw fishes[71] whereas Nematodes (roundworms) parasitism emerged independently at least five times in terrestrial vertebrates[72]. Helminth parasites where thus present during the key periods in the evolution of the vertebrate immune system[70, 73] (Figure 1.6) and exerted a strong pressure for human genetic adaptation to local environments[74]. This led to the co-evolution of an intricate host-parasite relationship where helminths assumed an essential role in the development of tolerance and immune regulation pathways[69,25].

Epidemiological studies support a correlation between the decrease in soil-transmitted helminthiasis and the occurrence of several immune related disorders, including MS, IBD and allergies[75]. All these disorders share a complex aetiology and several genetic risk loci (Table 1.4), suggesting that similar mechanisms might be involved.

According to the helminth therapy concept, reintroducing helminths-derived stimuli to the immune system might help restoring the aberrant immune responses occurring in immune related diseases.

In the following chapter, I will give an overview of the different helminth species, address the use of helminth therapy for organic diseases and review the mechanisms of helminth-mediated immune-modulation with a special focus on inflammatory bowel diseases.

1.3.1 Parasitic Helminths

Helminth parasites are large multicellular worms that colonise the intestine or the blood vessels of their host. Human helminthiasis belong to the neglected tropical diseases and infect about hundreds of millions of people worldwide[76].

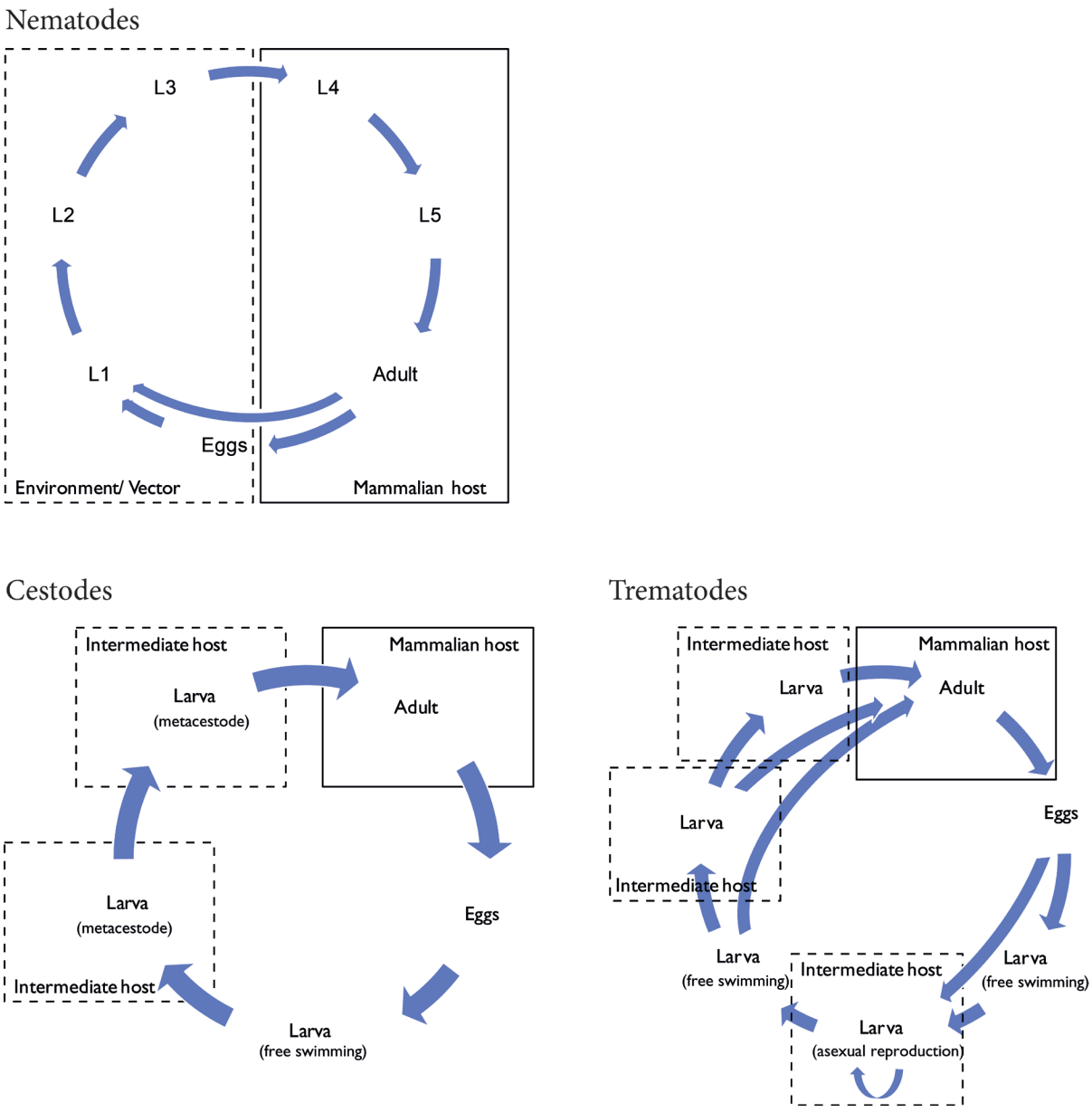

Figure 1.7: General life cycles of Nematoda, Cestoda and Trematoda. Plain squares represent the definitive hosts harbouring the adult helminths; dotted squares the intermediate host harbouring larval stages.

Generally, parasitic helminths have a slow maturation time and exert limited pathogenicity. The different helminth species are classified into cestodes and trematodes that belong to the phylum

Plathyhelminths and nematodes that constitute the phylum Nemathelminthes. Low burden helminth infections usually lead to chronic infection that can last several years and usually cause only a reduced morbidity. In order to colonise a great number of different environments, helminths evolved the capacity of maintaining homeostasis under a variety of environmental stress factors. Most species spend their life-cycle stages in different environments and need to adapt as they mature (Figure 1.7).Key for the survival is the secretion of bioactive molecules involved in immune evasion, extra corporal digestion, moulting and tissue colonisation. Helminths also expel waste and products that cannot be digested by means of excretion. Since the distinction between the secreted and excreted molecules is complicated, they are collectively termed E/S products[77]

Nematodes are non-segmented cylindrical worms and are the most numerous helminths in term of species diversity. The larval stage undergoes 4 moults to reach the adult form. Adult nematodes produce eggs that embryonate in utero or in the external environment[78]. The infectious stage is usually present in contaminated soil and thus the most common nematodes are known as soil-transmitted helminths (STH)[79]. The nematodes of the genus Trichuris, that represent the focus of this thesis, are discussed in chapter 1.4.3.

Cestoda are flat, ribbon like, hermaphroditic helminths (tapeworms). The adults are confined to the digestive tract of their hosts since they do not possess an alimentary canal. On the other hand, larvae occupy a greater variety of intermediate niches varying from insects to mammalian hosts. The transmission between different hosts occurs via ingestion of contaminated tissues. In the intermediate hosts the larvae invade the tissues and become encysted (metacestode form). Following ingestion by a definitive host, the larvae excyst and develop to the adult form. Among the human parasites five species belong to the Teniadee family and one to the Hymenolepidae family.

Finally, Trematoda, or flukes, parasitize a wide range of hosts and undergo complex life cycle stages with both sexual reproduction and asexual reproduction. Most require a vertebrate or invertebrate host where the larvae proliferate and then colonise the digestive tract or its associated organ of vertebrates.

1.3.2 Helminth Therapy in Organic Diseases

To enhance their survival and reproduction in their mammalian hosts parasites have evolved a wide range of mechanisms to escape or divert the immune system. As we have seen in the previous chapter, different helminth parasites have different life cycle giving rise to a considerable variety in the interaction with the immune-system. Still, some common patterns can be observed. An increasing number of studies suggest that the immunomodulation exerted by helminths can be used to counteract an aberrant immune response and treat organic diseases. An ideal therapeutic helminth should have – besides the essential immune-modulatory potential- a limited pathogenicity in the human host and a simple life cycle with no dissemination (Table 1.6).

Table 1.6: The ideal therapeutic helminth.

No dissemination
Not pathogenic to human
Self-limited infection
No replication inside the host
Not immediately infective
Easy administration
Effective anthelminthic available
Production in SPF conditions possible
Long term stability

Adapted from Elliot, 2007[80]

The infection with the therapeutic helminth should be non-productive inside the host to ensure a control over the amplitude of the immunomodulation. In case of complications, it is important that the parasite can be easily eradicated using standard anthelminthic drugs. Further, the production in a specific pathogen free environment is needed to avoid eventual co-infections.

As an alternative to live parasites, parasite extracts and parasite derived molecules can be used to avoid the risks of an uncontrolled infection (Table 1.7).

The following chapter will review the studies on helminth therapy for different organic diseases and discuss the possible mechanisms of action.

Table 1.7: helminth derived molecules with immune-modulatory properties

Molecule		***Parasite***	***Model tested***	***Mechanism***
rCKBP	chemokine binding protein	*S. mansoni*	air pouch, CXCL8- airway inflammation	↓chemokines induced infiltration of neutrophils
CPI-2	cystatine	*B. malayi*	-	↓endosomal/lysosomal proteases involved in antigen processing in a B-cell line, ↓MHC-II restricted antigen presentation
Cystatine	cystatine	*A. viteae*	OVA/grass pollen airway inflammation	IL-10 producing macs
Cystatine (cloned in E. coli Nissle)	cystatine	*A. viteae*	DSS colitis	↓IL-6 and IL-17A (locally), ↑Tregs, cathepsin inhibition: ↓Ag presentation on APC
rCsStefin-1	cystatine	*C. sinensis*	DSS colitis	↓ TNF in mLNs, ↑ IL-10 and IL-10+F4/80+ Macs in spleen and mLNs
rOv17	cystatine	*O. volvulus*	-	cathepsin L/S inhibition: ↓Ag presentation on APC, ↑ IL-10 by PBMC, ↓HLA-DR, CD86 on monocyte
MIF II	cytokine	*A. simplex*	DSS colitis	IL-10 by EC, DCs, and fibroblasts and TGFβ by fibroblasts
MIF-II	cytokine	*A. simplex*	OVA airway inflammation	IL-10 production and Treg, IEC-TLR2 dependent
MIF-II	cytokine	*T. trichiura*	-	↑ IL-10 and TNF from PBMC
Glycans	glycan	*T. suis*	-	↓LPS induced production of pro-inflammatory CKs (via CTL, MR) on DCs
LNFPIII	glycan	*S. japonicum*	EAE	↑ Th2 response
LNFPIII	glycan	*S. japonicum*	Flaky skin mice	Th2 priming DCs, via TLR4- NF-κB axis
ω-1	glycoprotein	*Schistosoma egg*	NOD mice	↑FoxP3 IL-4
11a 12b	PC	*A. viteae*	OVA induced airway inflammation	↓mast cell degranulation, CK production, Eos infiltration
PC moiety ES-62	PC	*A. viteae*	CIA	↓ IL-17/Il-22 in DC, γ/δ and CD4+ T (Th17) via MyD88 down-regulation (IL-1R/TLR axis)
PC moiety of ES-62	PC	*A. viteae*	OVA induced airway inflammation	↓ Th2 and Th17, ↑ Th1
rDiAg	polyprotein	*D. immitis*	NOD mice	↓ anti islet Th1 response, ↑IgE by B cells, CD40 agonist, NO by Macs
rDiAg	polyprotein	*D. immitis*	Murine spontaneous abortion	↓ IL-4, IL-23 and TNF
Mr85 Mr105	porin	*T. muris*	-	ECM degradation (syncytial tunnel formation)
TsTCI	serpin	*T. suis*	-	Inhibits Neutrophil elastase, the mast cell protease mMCP-1 and Cathepsin G.
asparaginyl-tRNA synthetase (rBmAsnRS)	tRNA synthetase	*B. malayi*	T cell transfer colitis	CD8+ response

APC: antigen presenting cell, CIA: collagen induced arthritis; EAE: experimentally induced autoimmune encephalomyelitis; ECM: extracellular matrix; LPS: Lipopolysaccharide; mLN: mesenteric lymph nodes; NOD: non-obese diabetic, PC: phosphorylincholine (references in text).

1.3.3 Helminth Therapy in Allergy

Already in the first descriptions of hay fever (initially called catarrhus aestivus), the highest incidence was reported in upper class patients[81]. Despite this early recognition, it took almost three decades until the scientific community started realising that these income and regional differences could be ascribable to the occurrence of parasitic infections. The turning point was the discovery of IgE in 1968[82] that was found to be up-regulated in both allergy and parasite patients[83] The observation that patients suffering from hay-fever entered remission following Ascaris infection, led Preston to speculate that the atopic syndrome could be a consequence of good hygiene and the lack of nematode infections[84].

Whilst studying the role of IgE in allergic reactions, Jarret and colleagues[85] noted that in rats infected with *N. brasiliensis*, subsequent passive skin test for systemic anaphylaxis and skin test reaction were strongly reduced. Confirming these observations, patients infected with different parasitic species were barely affected by passive skin sensitisation[86].

Shortly after, following his epidemiologic studies on asthma in rural and urban Gambia, Godfrey firstly suggested the treatment of allergic disorders with preparations of parasite antigens[87]. Turner promptly followed his advice and was successful in self-treating his allergic rhinitis by self-infection with 250 *N. brasiliensis* larvae at regular intervals[88] (yet in a later article he reported severe gastro-intestinal symptoms[89]).

Ten year later, Emanuel listed the change in immunological status caused by the removal of parasitic infestations as one of the possible causes for the post-industrial increase in the prevalence of allergic rhinitis[90]. Afterwards, several epidemiological studies confirmed the preventive effect of parasitism in the development of allergies[91;92].

Several mouse studies have shown a protective effect of helminths against the development of allergies. In a mouse model of food allergy, intragastric administration of peanut antigen and cholera toxin induces the production of Ag-specific IgE and systemic symptoms of anaphylaxis. An IL-10-dependent reduction in the allergic response occurs in *H. polygyrus* infected mice, where the down-regulation of Ag-specific IgE is associated with a reduction of Ag-specific T cells secreting IL-13[93]. *H. polygyrus* also protects from OVA-induced lung inflammation, although it is not clear if IL-10 is necessary or if alternative pathways can be induced[94;95]. IL-10 appears essential in the prevention of OVA-induced lung inflammation by *N. brasiliensis*[96] and in the protection from systemic fatal anaphylaxis by *S. mansoni*, where an increase in IL-10 producing B cells inhibits the IL-4 allergic response[97]. The important role of IL-10 is supported by studies with the filarial derived Cystatin that appears to supress both OVA and grass pollen allergen induced airways inflammation via induction of IL-10 producing macrophages[98;99].

Another filaria derived product, the glycoprotein ES-62, protects from OVA-induced inflammation by reducing Th2 and Th17 responses and inducing Th1 responses[100]. ES-62 is a large molecule, highly glycosylated and decorated with a phosphorylcholine (PC) modification that possesses immune-modulatory properties. PC was used as a template to design small, low weight analogues that were synthesized and tested for immune-modulatory properties[101]. Two of these molecules 11a and 12b, inhibit mast cell degranulation and cytokine production and prevent OVA-induced airway inflammation and eosinophil infiltration in the lungs[102].

The protective effects of *O. dentatum* extracts in a birch pollen allergy model and of *T. suis* E/S products in the OVA model further confirm that an infection with live parasites is not essential for the prevention of allergy development[103;104]. Schistosoma produce chemokine binding proteins (r-smCKBP) that bind CXCL8 and reduce neutrophil infiltration in an air pouch model and in CXCL8-induced airway inflammation. Through is blockade of Neutrophils' infiltration, r-smCKBP is also protective in a contact hypersensitivity model[105].

These and other studies demonstrate the protective effect of helminths and helminth products against the development of allergies, yet whether helminths ameliorate an already established allergy is less clear. *N. brasiliensis* infection in sensitized rats does not prevent a subsequent hypersensitivity response[106]. *N. brasiliensis* also fails to protect mice from an established OVA allergy and from OVA anaphylaxis[96].

In contrast, *Strongyloides venezuelanis* infection following an OVA challenge protects rats against AHR induction two days later, when the larvae migrate through the lungs[107]. A therapeutic effect is also exerted by *H. polygyrus* in OVA sensitized mice[95]. Finally, *A. simplex* produces a MIF-homologue that can suppress airway inflammation in mouse by inducing IL-10 production and Treg recruitment; this effect is strongly dependent on the expression of TLR2 by lung epithelial cells[108].

Following the initial success of helminth therapy in inflammatory bowel diseases, two double blind, randomized placebo controlled studies were performed in allergic rhinitis patients using *Necator americans* larvae [109;110] or *T. suis* ova (TSO)[111-113]. Yet, both studies failed to provide sufficient evidence on the efficacy and tolerability of helminth therapy in allergic rhinitis and a recent Cochrane review concluded that more preclinical studies should be performed to provide sufficient evidence on the efficacy of helminth therapy in the management of allergic rhinitis [114].

1.3.4 Helminth therapy in Multiple sclerosis

In 1966, an epidemiological study reported a correlation between the aetiology of multiple sclerosis (MS) and the level of sanitation in Israel[115]. The author proposed that increased levels of sanitation might postpone the encounter with the "causing infectious agent" later in life, when the central nervous system is more susceptible to demyelination.

After the first successful experiments in IBD and diabetes, two studies investigated a treatment with *S. mansoni* ova in the murine EAE (experimental autoimmune encephalomyelitis) model[116]. Ova treatment decreased the incidence and delayed the onset of EAE in wild type mice, but not in STAT6 deficient animals. Further, it reduced MOG-specific IFNγ, NO, and TNF production[116;117]. A soluble egg antigen from *S. japonicum* is also protective: among its components a glycan, Lacto-N-fucopentaose III (LNFPIII), is sufficient to reduce EAE severity and induce a Th2 profile[118]. Similarly, *F. hepatica* promote tolerogenic DCs that induce parasite specific Treg cells. In turn, Treg produce IL-10 and TGFβ that supress Th1 and Th17 responses[119]. Moreover, *T. crassiceps* limits the production of IL-17 and TNF and the migration of T cells to the CNS[120].

A set of clinical studies[121; 122] compared 12 MS patients with a mild, asymptomatic intestinal parasitism with 12 uninfected MS patients over a period of 7.5 years. The infected MS patients had a better clinical and radiological outcome. This protection was associated with the induction of Tregs, anti-inflammatory cytokine secretion, increased expression of TLR2 on B cells and DCs and of the MHC Class Ib molecule CD1d on IL-10 producing B cells.

Both the MS-protection and the anti-inflammatory pattern reverted in four patients receiving anti-helminth treatment. Additionally, two separate non-randomized studies have evaluated the use of *T. suis* ova (TSO) in the treatment of newly diagnosed relapsing remitting MS and secondary progressive MS[123,124,125]. In both studies TSO induced a modest shift toward a Th2 response and a mild eosinophilia.

Unfortunately, TSO led to a transient reduction of the MRI lesions only in newly diagnosed MS whereas an effect on severe MS patients has not been shown to date.

1.3.5 Helminth therapy in Rheumatoid Arthritis

The first correlation between parasite and rheumatoid arthritis (RA) protection was the incidental finding that infection with the nematode *Syphacia oblevata* protects male rats (female rats show small differences only) from the development of complete Freund's adjuvant induced rheumatoid arthritis[126]. Following anti-parasitic treatment with piperazine the rats started manifesting the adjuvant disease. Parasite infections in other murine RA models lead to contrasting outcomes.

Schistosoma infection reduces the severity of subsequent collagen induced arthritis (CIA) but exacerbates an already established CIA[127;128]. Further, *Hymenolepis diminuta* worsens a subsequent induced polyarthritis[129] whereas *Taenia crassiceps* infection has no effect on the development of RA induced by adoptive OVA-specific T cell transfer[130].

More promising appears the treatment of collagen-induced arthritis severity with the filaria-derived glycoprotein ES-62[131]. The PC moiety of ES-62 possesses immune-modulatory activity[132] and targets, DCs, γ/δ and $CD4^+$ T cells to modulate the IL-17/IL-22 producing network[133]. The mechanism of

action appear to involve the down regulation of the adaptor MyD88 in Th17, mast cells and macrophages that lead to an inhibition of TLR/IL-1R signalling[134;135].

A small molecule analogue of the ES-62 PC moiety (11a) was successfully employed to prevent CIA in mice and caused a down-regulation of the IFNγ and IL-17 responses[101].

Early studies in mice[136;137], showed that an *A. suum* infection or treatment with *A. suum* extracts can reduce the delayed type hypersensitivity to unrelated antigens through modulation of B and T cell responses. Similarly to ES-62, an extract from *A. suum* delivered parenterally or orally protects against the development of zymosan-induced arthritis in rats and therapeutic effect against CIA in mouse. *A. suum* extract reduced the levels of nitric oxide (NO), IL-1β (in rats), and IL-10[138].

1.3.6 Helminth therapy in Psoriasis

Flaky skin BALB/c mice have a spontaneous skin phenotype that closely resembles human psoriasis and they develop skin lesions as early as 5 weeks of age, in humans, mutations in the gene responsible for this phenotype (*TTC7*) also result in a severe form of very early onset IBD. Treatment with the Schistosome LNFPIII prevented the development of skin lesions in 77% of the mice[139]. LNFPIII signals via TLR4 causing a rapid and transient NF-κB activation that leads to the activation of DC primed to induce a Th2 phenotype[140].

1.3.7 Helminth Therapy in Transplant Rejection

BALB/C mice were infected with *T. spiralis* larvae per oral (*p.o*). 23 days prior to a body-skin graft from C57BL6 donors. Oral infection significantly prolonged the length of transplant retention from 10.7 days to 24.5 days. Timing is crucial, as an infection performed 7 days before transplant, has a milder effect[141]. Injection of a soluble larval extract is sufficient to delay rejection, supporting a role of an E/S product[142].

In humans, Echinococcus infection might cause the development of hepatocellular carcinoma that eventually requires liver transplantation. Since the usual immunosuppression is inefficacious and leads to the exacerbation of the parasitic disease, milder immunosuppression regimens are used. Yet, the transplants are usually well tolerated and the rejection rates are low[143]. This effect has been reported in mouse models of both liver and heart transplants, indicating a systemic effect[144].

N. brasiliensis or its extract prolonged the survival of rat kidney and heart in both rat and mouse models and reduced the infiltration of $CD8^+$ and to minor extents of $CD4^+$ lymphocytes. *N. brasilienis* also diminished the allospecific cytotoxicity of spleen lymphocytes and polarized toward a type 2 effector response[145]. The survival of heart allografts persists well behind the length of infection, further supporting a type 2 polarisation at the time of allograft-antigen presentation rather than a direct effect of the parasite.

Schistosoma infections also appear to prolong the survival of skin-allograft in both humans[146] and mice[147]. Interestingly, the protective effect is only observed in mice infected 60 days prior to the transplant, suggesting a role for the late Th2 response induced by Schistosoma. A recent study showed that infection of the recipients with *H. polygyrus* protects mice from graft versus host disease following a bone marrow transplant. *H. polygyrus* enhances the survival potential of TGFβ–generating recipient Tregs after body irradiation and favoured a TGFβ–dependent increase of donor Tregs[148].

The mammalian foetus represents a semi allograft within the maternal uterus since it expresses (as do the extra embryonic membranes) paternal MHC transplantation antigens. From this point of view, pregnancy loss can be seen as a failure of tolerance induction at the maternal–foetal interface. Women suffering from repeated pregnancy loss have higher serum of IL-17 and IL-23 levels and increased expression IL-17, IL-23, and retinoid orphan receptor C (RORC) in the decidua in comparison to women with normal pregnancy[149]. In addition, they have a decreased number of Tregs in peripheral blood to nonpregnancy levels[150].

Using a murine model of spontaneous abortion[151], Shihoko et al. could show that a filarial polyprotein significantly reduces the foetuses resorption rate from 42.9% in the control mouse to 11.1%. Interestingly, the reduction of pregnancy losses occurred in the absence of a Th2 CK up regulation and was characterised by diminished serum levels of IL-4, IL-23 and TNF whereas IL-17 levels were unchanged[152].

1.3.8 Helminth Therapy in Diabetes

The parasite-induced shift of the T helper response was discovered in the last decade of the 20th century. Knowing that several auto-immune disorders were characterized by an aberrant Th1 response, Cooke and colleagues decided to test *S. mansoni* to prevent or treat insulin dependent diabetes mellitus in non-obese diabetic (NOD) mice[153].

S. mansoni larvae first induce an initial Th1 response. 5-6 weeks later, the larvae start laying eggs that drive a marked Th2 response. Cooke showed that infecting 5-7 weeks old NOD mice with Schistosome larvae reduced the incidence of diabetes (described as blood glucose levels above 12 mmol/l). Further, diabetes was also prevented by injection of Schistosome eggs in 5 weeks old mice, highlighting the importance of the Th2 response.

Infection with a viable parasite is not necessary for the prevention of diabetes in NOD mice; filaria derived factor injected in 6-week-old NOD mice completely prevent insulitis and diabetes development by impairing the anti-islet Th1 cell response[154;155]. A similar experiment with soluble antigen from *S. mansoni* larvae or eggs, highlighted the importance of treating NOD mice at 4 weeks of age, when islet antigens presentation in pancreatic lymph nodes gradually cause the infiltration of

APC and lymphocytes[156]. *S. mansoni* eggs products induce Tregs, aaMacs, Th2 responses, and increase the numbers of NKT that are usually reduced in NOD mice[157]. Supporting a role of NKT, presentation of eggs glycoconjugates by dendritic cells (DC) to CD1d-restricted cells such as NKT is important for the induction of an adequate Th2 response[158;159]. Diabetes in NOD mice is also prevented by injection of the Schistosoma egg glycoprotein ω-1 that induces both FoxP3 and IL-4[160]. A preventive Th2 response is also observed in NOD mice infected with the gastrointestinal helminths *T. spiralis* and *H. polygyrus*[161]. *H. polygyrus* protection from diabetes also occurs in a Th2 deficient environment through an IL-10 dependent mechanism[162]. Rather than inducing a Th2 response, excretory/secretory products of the helminth *Fasciola hepatica*, supress the auto-antigen specific production of IFNγ and increase the numbers of IL-10 secreting B cells an aaMacs that might inhibit the initiation of auto reactive T cell responses[163].

Although a multitude of studies showed a protective effect of helminths or their derivatives in the prevention of diabetes in NOD mice, their clinical relevance is not clear. In fact, it is relatively easy to prevent diabetes in NOD mice even with nonspecific interventions [164]. Another common model of type 1 diabetes in rodents implies the administration of streptozotocin (STZ) a cytotoxic glucose analogue that causes the destruction of β-cell. The pathology caused by multiple low doses of STZ is immune-mediated and can be reduced by infection with *H. polygyrus* [165] that protects pancreatic islets and reverses the increase of pro-inflammatory mediators in a STAT6 and IL-10 independent manner. In contrast, the hyperglycemia induced by single high dose STZ is immune-mechanism independent and is not affected by *H. polygyrus* infection.

1.3.9 Helminth Therapy in Autoimmune Hepatitis

Autoimmune hepatitis (AIH) is a chronic hepatocellular inflammation and necrosis that, if left untreated, predisposes to cirrhosis and to hepatocellular carcinoma.

In the mouse AIH model, *i.p* injection of Concavallin A leads to a massive Th1 lymphocytes activation and infiltration into the liver parenchyma. Treatment with the adult worm extract from *A. suum* (Asc) 30 minutes before Concavallin A reduced the extent of liver damage, induced a type 2 response and increased the survival rate form 38% to 100%. Instead, treatment 2h after Concavallin had only a limited efficacy. Unfortunately, both the preventive and the therapeutic treatment with Asc exacerbated liver fibrosis, an adverse effect that might limit the translation of Asc use into the clinic both for AIH and for other immune related diseases[166].

1.3.10 Helminth Therapy in *H. pylori*-induced Gastritis

Helicobacter pylori is a component of the gastric flora in approximately 50% of the world population. It is well established that chronic helicobacter infection causes gastritis and peptic ulcer disease and predisposes to metaplastic changes.

In mouse, pre-treatment with *H. polygyrus* appears to limit the extent of *Helicobacter felis* induced atrophy of the glandular epithelium, reduced mucosal hyperplasia and mucosal metaplasia 16 week post- infection[167]. A similar study in the Mongolian Gerbils model of helicobacter-induced gastritis showed that, at 21 weeks post-infection, gastritis indices were lower in co-infected gerbils and were inversely proportional to worm burden.

However, the protective effect was lost by week 42 and the initial protective overexpression of anti-inflammatory mediators was transient and followed by a down regulation at week 42[168]. Male insulin gastrin (INS-GAS) transgenic mice suffer from a moderate chronic gastrinemia and develop gastric intraepithelial neoplasia 5-7 months after *H. pylori* infection.

A recent study showed that INS-GAS mice preventively infected with *H. polygyrus* were less susceptible to *H. pylori* induced gastric atrophy and dysplasia and were resistant to gastric colonisation with enteric microbiota, a phenomenon that is thought to contribute to gastric-carcinogenesis in humans and mice[169].

1.3.11 Celiac Disease

Preliminary trials were performed in 10 celiac disease patients in remission. In a first small double blinded placebo controlled trial, patients were treated with infective *N. americanus* larvae[170]. In a subsequent crossover study, 7 of patients previously enrolled in the control group received the infected larvae[171]. Although the symptom severity following gluten challenge remained unchanged, *N. americanus* reduced IFNγ and IL-17production by intestinal tissues. Additionally, the response to gluten challenge acquired a Th2 profile.

A subsequent open label trial showed that a combination of *N. americanus* and desensitization immunotherapy by micro challenge with deamidated gluten prevent histology and serology changes following gluten challenge[172]. The study enrolled 12 patients with diet-managed celiac disease. Among them, two subjects withdrew after microchallenge with 1g gluten. This promising study calls for a larger, double-blinded trial testing the efficacy and safety as well as the relative importance of *N. americanus* infection and gluten microchallenge[172].

1.4 Helminth therapy in IBD

1.4.1 Animal Models of IBD

Although it is impossible to reproduce the complexity of IBD in a laboratory animal, colitis models have contributed largely to our understanding of the human disease. Table 1.8 summarizes the criteria that a relevant IBD model should possess.

A relevant IBD model should resemble the human pathology both in its clinical manifestations and in its pathogenicity. Further, the induction protocol should be simple and the outcomes highly reproducible[173] (Table 1.8).

Table 1.8: Criteria for a relevant IBD model

easy induction
similar clinical manifestations
similar pathogenesis
respond to establish therapy
reasonable time course
reproducible results
available species with well-defined strain

Adapted form Bylund-Fellenius, et al, 1994[173]

Most models are performed in rat and mouse and can be divided according to the induction method (Figure 1.8). Chemically induced models are widely distributed, cheap and simple models that imply the application (oral or rectal) of a chemical compound. Congenic models include mouse strains that spontaneously develop intestinal pathology (colitis and ileitis). Genetically engineered models comprise knock-out or transgenic mice that develop colitis or ileitis spontaneously.

Adoptive transfer models require the transfer of T cells (usually $CD4^+$ $CD45RB^{high}$ T cells) into an immunodeficient recipient. Finally, spontaneous colitis is observed in cotton-top tamarins kept in captivity[174]. The different models are summarized in Figure 1.6 and the most common are described in further details in this section.

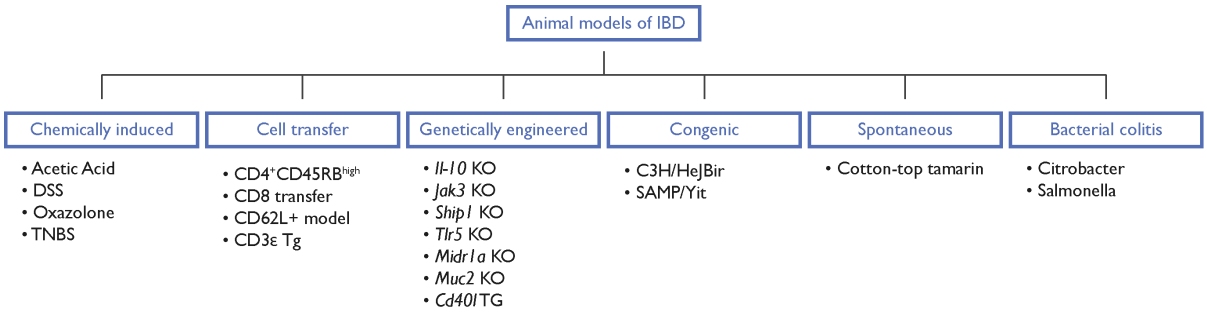

Figure 1.8: Animal models of IBD, classified according to the method of induction. The listed models are representative of the induction categories.

1.4.1.1 DSS

The chemical induction of colitis with dextran sodium sulphate (DSS) is among the most used mouse-models of IBD. One reason is the simple induction protocol that implies the administration of DSS in the drinking water. Furthermore, this model does not require anaesthesia of the animals that is necessary e.g. when applying an enema. Acute and chronic colitis can be achieved by varying doses and dosage and other protocols are used to study colitis associated carcinogenesis.

Clinically, DSS colitis manifests with weight loss and diarrhoea that might lead to the death of the animals.

To date, the mechanism by which DSS penetrates into the cells is unclear[175]. In the colon, DSS associates with medium-chain fatty-acids and form nano-lipocomplexes that can disrupt epithelial permeability[176]. DSS leads to a disruption of tight and adherent junctions by inducing the loss of ZO-1 and claudins. It also exerts a direct toxic effect on IEC and causes erosions that can lead to the complete loss of surface epithelium[177]. Following the epithelial damage, luminal contents leak into the mucosa and activate innate immunity causing a Th1 response.

Bacterial products are recognized by TLR4 and TLR2 and other PRRs that direct the recruitment of neutrophils and regulatory T-cells to the intestinal site. In acute DSS colitis bacteria and bacterial products are essential for the initiation of inflammation[178]. Several studies reported higher number of bacteria in the mucosa following DSS administration[179], which is in agreement with the increased number of bacteria found in biopsies of UC patients[180].

Similarly to the changes observed in UC patients, an increased presence of pro-inflammatory species - such as Bacteroidaceae and Clostridium spp. and a decrease of the probiotic Lactobacillus species was found to correlate with acute and chronic colitis[179;180.] Adaptive immunity plays a secondary role since DSS induces colitis in SCID, *Rag KO* and other mice that lack T cells and B cells[181].

Single doses of DSS (2-4% weight/volume) induce acute colitis, with weight loss and signs of loose stool or diarrhoea, characterized by a Th1 Th17-mediated acute inflammation. The pathology features

hyperemia, ulcerations, submucosal edema, and histopathological changes which include disruption of crypt architecture, inflammatory cell infiltration, muscle thickening and goblet cell depletion.
Multiple, lower doses of DSS (1-2% weight/volume) cause chronic colitis and in some cases lead to the development of malignancy. Chronic colitis leads to a Th2-mediated inflammatory response, with an increase in IL-4 and IL-10 and a concomitant decrease in TNF, IL6, IL-17[182].

1.4.1.2 TNBS

Trinitrobenzene sulfonic acid is a haptenising compound that is applied intra-rectally in an alcoholic solution. TNBS haptenizes autologous or microbial proteins and renders them immunogenic. This leads to a delayed hypersensitivity response and to the development of a Th1/Th17 mediated immune response. In mouse, TNBS causes a progressive weight loss, diarrhoea and faecal blood. TNBS colitis exhibits a diffuse colonic inflammation with transmural leukocyte infiltration (in particular $CD4^+$ T cells), oedema and ulceration.
For these characteristics, TNBS colitis is usually considered a CD like model[183].

1.4.1.3 Oxazolone

Intrarectal application of the haptenating agent oxazolone in ethanol damages the epithelium. The leakage of oxazolone and haptenized bacterial antigens initiate a self-limiting Th2 characterized by an increased IL-4 and IL-5 secretion.
Critical for the colitis development is the production of IL-13 by Th cells and NKT cells. The infiltration of NKT cells seems mediated by the gut-homing interaction between CCL25 and CCR9 that are up-regulated in both oxazolone colitis and human UC[184]. The induced histopathology also resembles the damages observed in UC patients[185] and is characterized by edema, infiltration of granulocytes in the superficial layers of the mucosa and ulceration of the epithelial layer.

1.4.1.4 Acetic Acid

Acetic acid is infused into the rectal lumen of a lightly anesthetized mouse, rat or rabbit and causes an initial mild epithelial necrosis and oedema that later variably extends into the lamina propria, submucosa, or external muscle layers, depending of the dose and dosage applied.
Other features include the infiltration of Neutrophils into the mucosa, vascular dilation and submucosal ulceration. Colitis is initiated by the acetic acid induced damage of the intestinal epithelium, which subsequently activates the immune response.
The pathology is probably related to an increased oxidative stress and the inflamed mucosa shows an increased ROS production and lipid peroxidation[186].

1.4.1.5 *Il-10 KO* Spontaneous Colitis

In 1993, Kühn et al reported that mice deficient in *Il*-10 (*Il-10 KO* mice) spontaneously develop a chronic enterocolitis[187]. *Il-10 KO* mice suffer from weight loss and anaemia and develop skip lesions that are typically most severe in the caecum and the proximal colon. The pathology includes increased neutrophil infiltration, erosions, transmural, mononuclear to granulomatous inflammation similar to CD, and diminished gut associated lymphoid tissue.

Intestinal microbiota are the trigger of colitis in the *Il-10 KO* model and the development of colitis is abolished when *Il-10 KO* nice are maintained under germ-free conditions. Interestingly, MyD88 expression in the mononuclear phagocyte compartment is needed for colitis development, suggesting a role for the signalling of commensals through the TLR-MyD88 axis[188].

Luminal antigens lead to a sustained production of pro-inflammatory cytokines that cannot be controlled due to the absence of IL-10.

1.4.1.6 Transfer Colitis

The adoptive transfer of $CD4^+CD45RB^{high}$ naïve T cells from healthy wild-type mice into syngeneic SCID recipients, lacking T and B cells, causes severe colitis in approximately 6 to 12 weeks[189]. In absence of $CD4^+CD25^+Foxp3^+$ Tregs cells, the naïve T cells are activated and expand in response to intestinal antigens and produce colitogenic Th1 and Th17 cells. Therefore, colitis can also be induced by transfer in *Rag KO, Cd3 KO, Nod KO* and *Tcrα Tcrβ KO* recipients[190]. The pathology features transmural inflammation, IEC hyperplasia, leukocyte infiltration, crypt abscesses, and epithelial cell erosions[191]. Transfer colitis models are used to investigate the initiation of adaptive immunity in colitis and were crucial for the study of Tregs and their role in maintaining homeostasis into the intestinal mucosa[192].

1.4.1.7 Citrobacter Colitis

The Gram-negative, non-invasive mouse pathogen *C. rodentium* has been extensively used to investigate the contribution of intestinal commensals and pathogens to the development of IBD[193]. *C. rodentium* colonises the caecum and the colon causing a transient and self-resolving mild colitis with crypt hyperplasia, loss of goblet cells and infiltration of mononuclear immune cell infiltration[194]. The infection changes the composition of both adherent and luminal microbiota and increases the numbers of Proteobacteria, Clostridia, whilst reducing the abundance of Lactobacillus spp.[195].

C. rodentium induces a chronic colitis in mice with a compromised intestinal barrier and in immunocompromised mice (e.g *Rag KO*). Studies in KO mice revealed the importance of the TLR2/MyD88 axis for the maintenance of the epithelial barrier function[196] and of B cells for the recognition and clearance of the pathogen[197].

1.4.1.8 Spontaneous Colitis in the Cotton-Top Tamarin

Captive cotton-top tamarins (*Saguinus oedipus)* develop a colitis featuring periodic flares, whose histological appearance involves diffuse mucosal inflammation and crypt abscess formation.

The pathology resembles UC and presents an increase in various inflammatory mediators such as prostaglandins, interleukins and myeloperoxidase. Chronic colitis also leads to the development of colorectal cancer rendering the tamarin a good model for both diseases.[198].

1.4.1.9 Post-Weaning Syndrome

Young piglets often develop a post-weaning intestinal inflammation characterized by an increase of both $CD4^+$ and $CD8^+$ T cells as soon as 2 day post weaning. The pathology is accompanied by an increased expression of IL-1β, IL-6, and TNF and an increased permeability in the intestine[199].

1.4.2 Helminth Therapy in Animal Models of IBD

The first animal studies in IBD were performed with some delay with respect to the early pioneer studies in RA, allergic rhinitis and MS.

In 1991, Urban noticed that a concurrent enteric helminth infection could modulate the inflammation and gastric immune responses induced by *H. pylori* [200]. One year later, Elliot and Weinstock showed that exposure to *H. polygyrus* reduces intestinal inflammation in *Il-10* deficient mice, starting a long series of studies with different helminths in different models of IBD[201].

The intestinal nematode *H. polygyrus* has now shown protective and therapeutic potential in several models of murine colitis including chemically induced colitis (TNBS and DSS), T cell transfer colitis and antigen driven colitis. However, the beneficial modulation of the immune response exerted by *H. polygyrus* also dampens protective responses such as the recruitment of intestinal phagocytes. This leads to impaired resistance to *C. rodentium* and Salmonella and enhances the infectious colitis[202;203.]

The results with the cestode *H. diminuta* are less clear. Administration reduces the severity of a subsequent DNBS colitis in mice via the induction of alternatively activated macrophages, IL-10 producing T cells and TGFβ producing regulatory B cells[204]. In contrast, preventive or therapeutic administration of *H. diminuta* larvae to DSS mice failed to improve the histological damage, despite a shift of the immune response toward a Th2 response[205]. The enhanced Th2 response and the consequent eosinophilia are probably the reason for the detrimental effect of *H. diminuita* observed in the Th2-biased model of oxazolone colitis[206].

The trematode *Schistosoma* was first tested in a TNBS model of colitis. BALB/C mice injected intraperitoneally with freeze-killed *S. mansoni* eggs 4 days prior to the induction of colitis show a STAT-6 dependent attenuation of colitis, with an augmented Treg and Th2 response[207]. Freeze-killed *S. japonicum* eggs also protect from TNBS colitis preventing the disruption of tight junctions and

reducing the TNBS induced TLR4 and NOD2 up-regulation, leading to a Th2 polarisation[208]. The integrity of the egg is not a requisite for the protective effect: intraperitoneal injection of *S. japonicum* soluble egg antigen protects mice from T cell transfer colitis by reducing the Th1 and Th17 responses in favour of TH2 responses[209]. In contrast, injection of either live eggs or soluble eggs antigen fail to protect from acute DSS colitis despite inducing a Th2 shift[210,211]. Also, in the DSS model, egg-laying infections with *S. mansoni* have no preventive effect and increase the symptoms of the colitis. The noxious effect is ascribable to the laid eggs, as non-productive infections with male larvae attenuate the DSS colitis and diminish both Th2 and Th1 cytokines levels.

Another nematode, *T. spiralis,* induces a Th2 response and attenuates a subsequent TNBS colitis[212]. As for other parasites, protection is reproduced using a *T. spiralis*-derived antigen[213], with a down regulation of iNOS expression and IL-1β production and an up-regulation of colonic Th2 and aaMacs markers. In vitro experiments support an alternative activation of macrophages, that in response *to T. spiralis* antigens upregulate *Arg1, Mr, Ym1* and *Il-10* in a STAT6 dependent, IL-4R independent manner[214].

Recent studies tried to substitute the live parasite with immune-modulatory parasite components or secreted products. The MIFII homologue produced by *A. simplex* that is protective in a murine asthma model also reduces the severity of a subsequent DSS colitis. *In vitro*, *A. simplex* MIF II induces in vitro expression of IL-10 by EC, DCs, and fibroblasts and TGFβ by fibroblasts[215].

The E/S products of the filarial nematode *A. vitae* also possess immune-modulatory activity. One of its components is a cysteine protease inhibitor (cystatine) that acts on macrophages and attenuates DSS colitis. Interestingly, *A. vitae* cystatin as well as cystatins from other helminthic parasites such as *Onchocerca volvulus*[216], *Clonorchis sinensis*[217] and *Brugia Malayi*[218] target antigen-processing cells whereas the cystatin produced by the free-living *C. elegans* does not[98]. Cystatins secreted by helminths during the mammalian phase, mimic the mammalian cystatins that modulate cathepsin activities and antigen presentation[218].

To directly target the site of inflammation, *A. vitae* cystatin was cloned in the probotic *E. coli* Nissle and administered to DSS feed mouse. Attenuation of colitis was associated with a decrease in macrophages, an increase of Tregs in the colon and a local decrease in the production of IL-6 and IL-17A[219].

The cystatin produced by *B. malayi* (CPI-2) inhibits endosomal/lysosomal proteases involved in antigen processing in a B-cell line and has been shown to inhibit MHC-II restricted antigen presentation in a peptide-specific manner[218].

Besides cystatins, *B. malayi* also produces the excretory/secretory protein asparaginyl-tRNA synthetase (rBmAsnRS) that attenuates T cell transfer colitis and induces a $CD8^+$ response[220].

1.4.3 *Trichuris suis* as an Ideal Therapeutic Helminth

Trichuris species are widespread helminths that parasitize several mammals including both wild and domestic animals. Among the different Trichuris, *T. trichiura*, *T. muris* and *T. suis* are the human, mouse and pig parasites. A Trichuris infection is initiated by the ingestion of embryonated eggs (Figure 1.9).

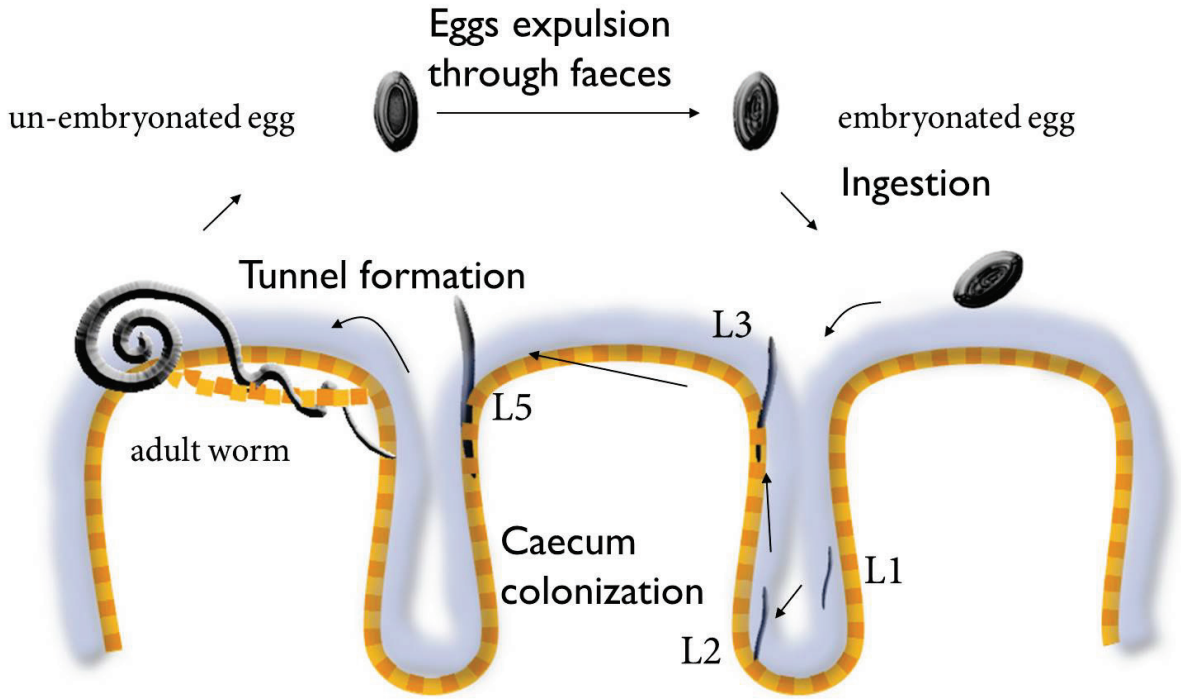

Figure 1.9: *Trichuris* life cycle. Infection occurs by the ingestion of soil contaminated with embryonated eggs. The eggs hatch in the caecum in response to bacterial stimuli. The larvae (L1) penetrate the caecum and proximal colon wall, dwell in the epithelial layer and mature to L2 stage. L3 larvae protrude the posterior end into the intestinal lumen, whilst the stichosome is anchored in an IEC tunnel. The larvae mature further to L5 stage and, after reaching sexual maturity, produce eggs that are expelled with the faeces. The eggs require an incubation time in the soil for embryonation to the infective stage.

Throughout its maturation in the hosts, Trichuris remains confined to the intestine and no dissemination to the lungs or other organs occurs. In a compatible host, the eggs hatch freeing L1 larvae that first attach to the villi and then move distally toward the caecal and proximo-colonic mucosa. L1 invade the intestinal epithelium on the crest of crypts, bringing with it luminal bacteria and antigens. The larvae remain embedded and develop in the mucosa gradually moving through

adjacent epithelial cells. At approximately 2 weeks post infection (in *T. suis* infected pigs) L3 larvae protrude the posterior end into the intestinal lumen, whilst the thin anterior end (called stichosome) is anchored in an inert tunnel composed of dead epithelial cells[221]. Within the tunnel Trichuris secretes digestive enzymes and immune-modulatory molecules and intakes nutrients through its absorptive bacillary cells.

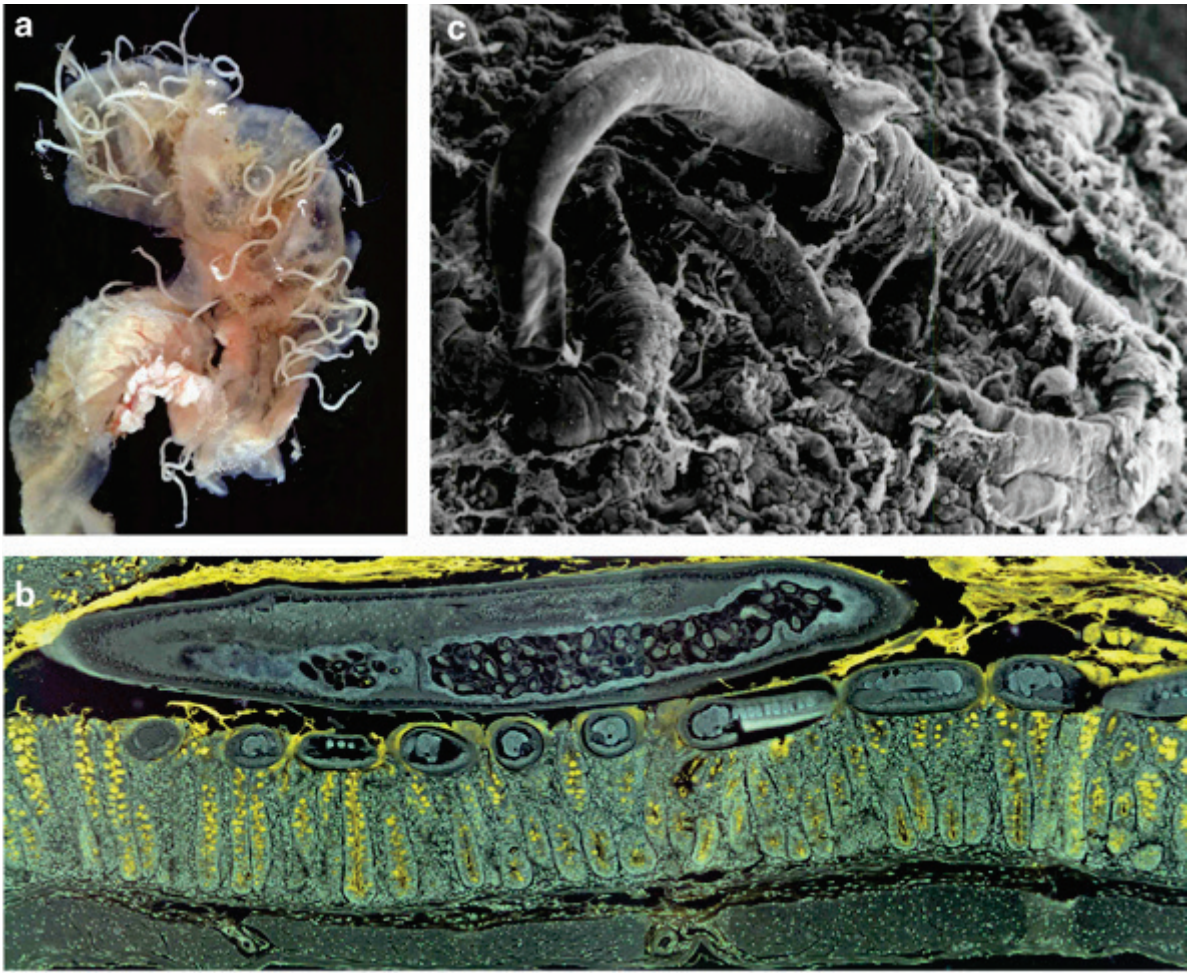

Figure 1.10: Colonisation of the caecal mucosa by *T. muris*. (**a**) Portion of the caecum of a *T. muris* infected mouse; the posterior ends of Trichuris extending into the lumen are visible (day 33 post infection). (**b**) Light micrograph of an adult female *T. muris* (day 42 post infection). Multiple transverse sections of anterior worm can be seen embedded within the epithelium, with transverse section of the posterior worm free in the lumen. Eggs are visible inside the worm uterus. (**c**) SEM of a portion of caecum on which the Trichuris stichosome is burrowed into the mucosal epithelium. From Artis, 2008[221].

The larvae mature locally to the L5 stage and, after reaching sexual maturity (*T. suis*: day 37 p.i/*T. trichiura*: day 60 p.i), produce eggs that are expelled with the faeces. Of note, the eggs are not immediately infective, and require an incubation time in a warm humid soil over a period of 3-6 weeks to embryonate[222]. Once embryonated, the eggs can be stored at 4°C for at least 1 year, without

compromising the larvae infectivity. The life span of the adult Trichuris depends on the species: *T. trichiura* infection can last 1 year whereas *T. suis* infection can last up to 2 years[222]. In humans, *T. trichiura* represents the third most common parasitic nematode and infects approximately 500 million people worldwide[223]. The morbidity of trichuriasis is low and the occurrence and severity of the symptoms depends largely on the worm burden. Trichuris causes usually causes mild gastrointestinal symptoms such as loose stools and abdominal discomfort. Still, dysentery, anaemia and rectal prolapse might occur in heavily infested patients[224].

Table 1.9: *T. suis* as an ideal therapeutic helminth

Intestinal colonisation, no dissemination
Not pathogenic to human
Self-limiting infection
Eggs are not immediately infective
Effective single-dose anthelminthic available
Production in SPF conditions possible
Long term stability of TSO

Trichuris spp, and more generally helminths, are usually species specific; for example *T. suis* ova fail to colonise the murine intestine. Occasionally, zoonotic transmission to humans can occur for some species including *T. vulpis* and *T. suis*[225;226]. The first reports of an experimental *T. suis* infection in human subjects resulted in a detectable infection after 5-8 weeks[227]. In two other cases cases, the infection was only transient and led to the production of eggs for approximately 10 and 16 days[228]. Only 11% of the recovered eggs could embryonate (indicating successful mating), whereas the reported rate for infections in pigs is 84-86%. Importantly, the subjects showed no adverse effects and no alterations in the leukocyte counts[227;228]. These findings confirm that man is not the normal host of *T. suis*. To our knowledge, there is no report of pathology attributable to *T. suis* in healthy individuals. Moreover, Trichuris infection can be eradicated with a single dose of antihelmintic drugs such as albendazole plus oxantel pamoate or mebendazole[229]. Lastly, *T. suis* ova for helminth therapy can be obtained from specific-pathogen free pigs, thereby minimising the possible risks associated to a coinfection with pathogenic viruses or bacteria. In light of these considerations, *T. suis* appears a safe helminth therapeutic candidate for IBD and other diseases (Table 1.9). The evidence supporting its efficacy and the immune-modulatory properties of the Trichuris spp. are discussed in the next chapter.

1.4.3.1 Trichuris in the Healthy Intestine

Whole genome sequencing revealed a high conservation of genes in Trichuris, we can thus speculate that different Trichuris species employ similar mechanisms to colonise the gut mucosa (Figure 1.11)[230;231].

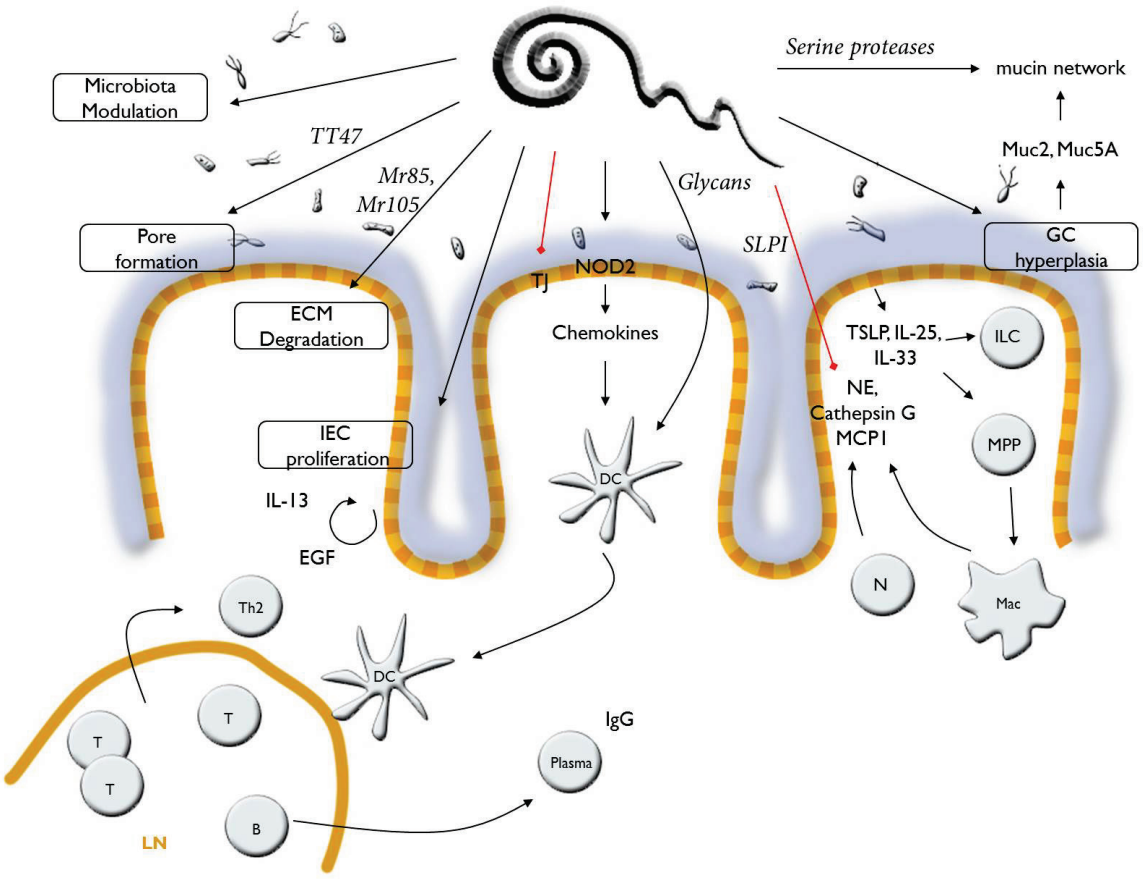

Figure 1.11: Modulation of the intestinal niche by Trichuris spp. Names in italics are molecules secreted by Trichuris. Red arrows represent inhibitory effects. B: B cells, DC: Dendritic cells, ECM: extracellular matrix, GC: Goblet cells, IEC: intestinal epithelial cells, ILC: innate lymphoid cells, LN: lymph nodes, Mac: Macrophages, MCPI: mast cell protease I, N: Neutrophils, NE: neutrophil elastase, Plasma: plasma B cells SLPI: serine leukocyte protease inhibitors, T: T cells, Th2: Type 2 T helper cells. Black arrows indicate stimulatory pathways, red diamond-tipped arrows indicate inhibitory pathways.

As the other helminths, Trichuris spp. secrete an E/S products to interact with its host. A recent analysis in *T. suis* has identified 618 genes encoding putative E/S products that constitute 10% of the whole transcript[231]. Proteases are highly represented in the secretome of Trichuris spp[232;230;231] and might be involved in the colonisation of the caecal mucosa. For example two serine proteases from *T. muris* (M_r 85 and M_r 105) possess ECM degrading activity and have been implicated in the formation of syncytial tunnel[233].

Other proteases are homologues known mammalian secretory leukocyte protease inhibitors[230;232] (SLPIs), that are not found in other helminth lineages and seem unique to the Trichuris spp. In mammals, SLPIs act as Neutrophil elastase inhibitors, anti-microbial peptides, and inhibitor of NF-κB in response to microbial products. Of note, SLPIs are strongly enhanced in inflamed versus non-

inflamed UC regions and SLPIs expression increases during resolution of DSS colitis[234;235]. SLPI seems to regulate the balance between host defence and tissue regeneration, and is crucial to mucosal healing of inflammation-mediated damage[236]. *T. suis* produces serine protease inhibitors (serpins) including the low weight TsTCI that inhibits a variety of proteases including Neutrophil elastase, the mast cell protease mMCP-1 and Cathepsin G. Other portions include channel and transporters such as the *T. trichiura* porin TT47, which induces ion-conducting pores in the lipid membrane and is involved in the formation of syncytial tunnels[233].

The *T. suis* transcriptome also includes homologues of macrophage migration inhibitory factor (MIF)[232], similar to the immune-modulatory proteins found in *A. simplex*[237]. A MIF homologue is also a component of a *T. trichiura* extract that stimulates the production of IL-10 and TNF from peripheral blood mononuclear cells (PBMC)[238]. *T. muris* is a natural mouse pathogen and is a widely used model to study Trichuriasis. The outcome of a *T. muris* infection depends on the mouse strain: most strains successfully expel *T. muris*, although with various efficacy. In contrast, strains that are more prone to a Th1 response, fail to mount a protective immune response and suffer long term chronic infections. Infections with low numbers of eggs (less than 100) usually cause no symptoms and lead to persistent infection whereas higher doses cause a more acute response that usually results in parasite expulsion[239].

Efficient hatching is a critical process for the establishment of Trichuris and is probably triggered by the optimum temperature and the increased bacterial numbers found in the caecal region. Treatment of mice with antibiotic reduces the worm-burden of a subsequent *T. muris* infection, but has no effect on an already established infection. Noteworthy, incubation of the embryonated eggs at 37°C with dead *E. coli* is sufficient to trigger hatching through the interaction with type I fimbriae. Bacteria lacking type I fimbria also trigger TSO hatching, suggesting the presence alternative recognition mechanisms[240].

Early studies have shown that parasite clearance is associated to a Th2 response, with the cytokines IL-4 and IL-13 playing a critical role. In contrast, susceptibility is associated with a Th1 response[241]. T cells play a crucial role in the control of Trichuris infections: T cell transfer form *T.muris* infected donors is sufficient to transfer immunity to naïve mice[242]. Yet, the interaction with the host immune system is more complex than a simple effect on T cell polarisation, with effects on both the innate and the adaptive immune response.

An acute *T. muris* infection causes goblet cell hyperplasia and an increased secretion of MUC2 and MUC5A, two mucins necessary for worm expulsion. In turn, *T. muris* secretes proteases that target MUC2 for degradation leading to the depolymerisation of mucin nets[243]. In resistant mouse strains, the host secretes higher levels of serpins that prevent the disruption of the mucous layer allowing

worm expulsion. In susceptible strains, chronic infections are accompanied by alterations of mucins modification such as lower D-GalNAc glycosylation and higher Sialylation. The altered mucin composition affects the intestinal microbiota composition and its interaction with the underlying epithelium[243]. Another expulsion mechanism observed in resistant strain is an increase in epithelial cells turnover. Epithelial proliferation functions as an epithelial escalator moving the parasite embedded in the epithelium from the bottom of the crypt to its top[244]. This process is IL-13 dependent and is enhanced by members of the epidermal growth factor (EGF) family such as Amphiregulin that is expressed by activated Th2 cells in response to *T. muris* infections[245].$CD4^+$ T cells and NK cells are the major producer of IL-13 in response to *T. muris*. Interestingly, IL-13 mediated protection is dependent on $CD4^+$ T cells and IL-18 signalling[246].

In susceptible strains, chronic infection causes a IFNγ, TNF mediated increase in IEC apoptosis and a down-regulation of the IEC turnover that on the one hand reduces worm expulsion and on the other hand might protect the host since it counters infection-induced IEC hyperplasia[247]. E/S products of *T. suis* enhance epithelial permeability by diminishing the expression of the tight junction proteins EMP-1 and Claudin-4. This effect is mediated by the glycan moieties and allows other components of the E/S product to cross the epithelium and affect cells on the basolateral side[248].

Further, in vitro studies have shown that *T. suis* E/S products elicit the production of IL-6 and IL-10 from IECs[249]. Epithelial cells of resistant animals also show enhanced levels of chemokines that lead to a rapid recruitment of DCs in the mucosa[248]. A prompt and increased recruitment of DCs might facilitate antigen sampling from the epithelium and the lumen and thus a rapid priming of T cells in the mesenteric lymph nodes (mLN)[250]. DCs differ from intestinal macrophages in expressing higher levels of MHC-II and CD11c and by lacking CD64. The murine gut DCs can be subdivided into $CD103^+$ $CD11b^+$ DCs, $CD103^+$ $CD11b^-$ DCs and $CD103^-$ $CD11b^+$ DCs[251]. In resistant strains, DCs express higher levels of the maturation markers CD80/86, MHC-II and CCR7 and possess lower endocytic activity than DCs in susceptible strains.

In vivo experiments with human monocyte derived DCs, revealed that glycans from Trichuris suis excretory/secretory antigen signals via C type lectins (CTL) and mannose receptors (MR) on immature DCs and supress LPS induced production of pro-inflammatory cytokines[252]. The recruitment of MHC-II^{hi} $CD11c^{hi}$ $CD103^+$ DCs (mostly $CD11b^+$) in response to *T. muris* is dependent on NOD2: The IECs in *Nod2 KO* animals are unable to produce chemokines essential for the recruitment of DCs to the colonic epithelium. *Nod2 KO* have a reduced T cell expansion, supporting the importance of DCs for T cell priming and gut homing. The defective T cell response might explain the delayed worm expulsion observed in *Nod2 KO* mice[250].

Despite these findings, *T. muris* infections in *Cd103 KO* mice showed that these cells are dispensable for the resistance to *T. muris* [253] suggesting the existence of alternative mechanisms.

Important mediators of the early response to Trichuris are the innate cytokines (alarmins) thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. *Tslp* is constitutively expressed by IEC in the caecum and colon. Exposure to *T. muris* excretory/secretory antigen (but also to viruses, bacterial pathogens, Th2 and Th1 cytokines) leads to an IKKβ mediated increase in TSLP production[248]. TSLP reduces the production of IFNγ, IL-12/23p40 and IL-17A by DCs and induces the production of type2 cytokines by CD4+ T cells[254]. In addition, TSLP mediate the accumulation of mast cells, and basophils that also contribute to the anti-inflammatory response[255].

IL-25 is constitutively expressed in strains resistant to *T. muris*, and its production increases in response to nematode infection[256]. IL-25 appears to promote a type 2 response mainly by reducing type 1 inflammation[257] and also stimulate type 2 cytokine production by a population of MHC-II^{high}, CD11c^{dull} Linn^{-} innate immune cells[258].

IL-33 is transiently produced in the caecum of *T. muris* infected mice in the early phase of the infection. IL-33 enhances TSLP production by IEC, induces Th2 polarisation, enhances the numbers of goblet cells and increases the production of IgE[259]. The production of type 2 cytokines in response to Trichuris is fast, although the parasite specific T cell response needs several days to develop. The fast response toward helminths is mediated by type 2 innate lymphoid cells (ILC) that are elicited by TSLP, IL-25 and IL-33. IL-25 simultaneously stimulate a distinct population of ILC named multipotent progenitor type 2 (MPP^{type2}) cells. MPP^{type2} accumulate in the mucosa following a *T. muris* infection; they induce an early type 2 response and can differentiate in to innate cells such as Macs, Mast cells and basophils capable of producing IL-4 and IL-13[260;261]. Mast cells and eosinophils are not essential for *T. muris* expulsion but they might play a supportive role[262]. IL-9 is a growth factor involved in the recruitment and survival of mast cells that is produced in response to *T. muris*.

Recent studies suggest that a distinct subset of Th2 cells produces IL-9 in response to TGFβ during *T. muris* infections and is required for parasite clearance[263]. Retinoic acid signalling via the retinoic acid receptor (RORyt) is essential for CD4^{+} T Cell effector responses and the generation of Th1 and Th17 and Th2 cells that reduced in the intestine of vitamin A deficient (VAI) mice. VAI mice also have less intestinal RORyt^{+} ILC3 cells and a diminished production of ILC-derived IL-22 and IL-17 that results in enhanced susceptibility and pathology to acute bacterial infection. In turn, they show increased numbers of intestinal ILC2 producing IL-13 that can compensate for the defective Th2 immunity and rescue the control of a *T. muris* infection[264]

The production of retinoic acid is reduced in a high-dose chronic *T. muris* infection due to a diminished numbers of ALDH^{+} macrophages and DCs. In acute infections the reduction in ALDH^{+}

cells is transient and is restored following the expulsion of the parasite. This indicates a role of retinoic acid in the modulation of immune responses[265].

A classic response to helminth infection is the differentiation and the accumulation of aaMacs. Despite this phenomenon, there is no clear evidence supporting an essential role of this cell population. In *T. muris* infections, aaMac, usually rare in the gut, increase in numbers at around 3 weeks post infection. Presence of arginase (ARG1) producing Macs is superfluous for the control of *T. muris* and no changes in the cytokines and antibodies responses occur in *Arg1 KO* mice[266]. *In vitro* studies with *T. suis* soluble products, revealed that in human macrophages the anti-inflammatory state is induced via TLR4[267]. Macrophages deficient in the phosphatase SHIP are more sensitive to IL-4 mediated aaMacs polarisation. Despite the increase in aaMAc numbers, *Ship KO* mice are susceptible to *T. muris* infections and develop a polarized Th1 response due to an increased production of IL-12p40 by macrophages, ephasizing the role of a proper macrophage response[268].

T. muris-specific antibody production in the mLN peaks two weeks post infection in both resistant and susceptible strains. Afterwards, the levels gradually decline in both susceptible and resistant strains, but remain above the naïve levels only in susceptible strains[269]. Underlining the importance of the antibody response, B cell deficient C57BL/6 mice fail to control *T. muris* infections, but can be rescued by reconstitution with splenic B cells or by treatment with Trichuris-specific IgG1 from resistant mice [270]. Of note, *T. muris* and other parasites (*H. polygyrus* and S. *mansoni*) can induce the production of IgE even in absence of the Ig μ- and δ-chains, suggesting the existence of an evolutionary old mechanism for the antibody response towards helminthic infections[271].

A modulation of the intestinal microbiota by Trichuris has been observed in pigs. *T. suis* infection alters the composition of the proximal colon microbiota and causes a shift in its metabolic potential affecting carbohydrate metabolism, lysine biosynthesis and fatty acid absorption[272].

In contrast, the effect of *T. trichiura* on the human intestinal microbiota is unclear[273;274]. An Ecuadorean study found no difference between the microbial composition in faeces from children infected with *T. trichiura* (n=17) and local uninfected controls (n=30). On the other hand, a similar study performed in Malaysia revealed an association between helminth colonization and increased microbial diversity in the faeces, which seems driven by Trichuris infection.

1.4.3.2 Trichuris Therapy in IBD Models

T. muris is a natural mouse parasite. Hence, it is not surprising that a concomitant infection in the *Il-10* KO model worsens the spontaneous colitis. *T. muris* exacerbates the Th1 and Th17 responses and increases the expression of the IL-13 decoy receptor (IL-13Rα2) resulting in a diminished IL-13 activity. IL-13Rα2 seems to prevent Th2 polarisation since *Il-10, Il-13rα2 double KO* mice are resistant to colitis and develop a less severe chronic colitis in response to *T. muris*[275].

T. muris also increases colitis severity in *Mdr1a KO* mice that spontaneously develop a Th1 colitis due to an epithelial barrier defect[276]. A concurrent *T. muris* infection also exacerbates colitis in the DSS model. Yet, when DSS colitis is induced after the expulsion of *T. muris* (from day 27 post infection), mice are protected and develop a milder colitis. In particular prior *T. muris* infection appear to favour re-epithelialisation and regeneration of the colonic mucosa[277]. *T. trichiuria* has shown positive effects in treating idiopathic chronic diarrhoea in macaque monkey. This small study showed an attenuation of colitis in 4 out of 5 treated animals, with the 5th requiring euthanisation due to progressive weight loss and diarrhoea. The attenuation of colitis coincided with a Th2 response and expression of genes characteristic a type 2 response. Further, *T. trichiuria* reduced the attachment of bacteria to the intestinal mucosa and induced changes in the microbiota composition[278].

1.4.4 Clinical Studies and Case Reports with Trichuris

The first small open label trial with *Trichuris suis* ova (TSO) was performed by the Weinstock group in 2003 with 4 CD and 3 UC patients (Table 1.10). The patients were treated with a single dose of 2500 TSO and were monitored regularly for 12 weeks. Importantly, no adverse effects were reported and no changes in laboratory parameters were observed. The results were promising: 6 of 7 patients achieved clinical remission at week 8 according to the IBD quality of life index. Yet, this effect was temporary and 3 of the patients relapsed within 4 weeks. In 2 CD and 2 UC patients the transient effect of TSO could be prolonged by repeated administration of TSO every 3 weeks for 28 weeks[279]. A later open-label trial enrolled 29 CD patients, with a mean CD activity index (CDAI) of 287.1±47.8, who received 2500 TSO every three weeks. At week 12, the CDAI improved in 22 patients (ΔCDAI>-100 points or reduction to CDAI<150) and 19 entered remission (CDAI<150). The improvement was maintained and the mean CDAI reached 99.9±35.6 at week 24[280]. A larger, randomized, double blind, placebo-controlled trial involved 54 UC patients with an UCDAI of 8.7±0.3. Of the 54 patients, 13/30 patients receiving 2500 TSO every 2 weeks experienced a response after 12 weeks ΔUCDAI >-4) versus only 4/24 patients receiving placebo[281]. Following this first phase, a crossover was performed by switching the therapy to the patient that still had UCDAI≥4. After additional 12 weeks, 56.3% of TSO treated patients responded relatively to 13.3% of the placebo patients. Unfortunately the number of patients included in this analysis is not clear, but the authors reported a significant effect[282].

Table 1.10: Clinical studies and case reports with *Trichuris suis* ova (TSO) and *Trichuris trichiura* ova in inflammatory bowel diseases, multiple sclerosis and allergic rhinitis.

Citation	Nematode	Dose and dosage	Baseline medication allowed	n/placebo	Kind of trial	Severity	Outcome	Adverse effects
				IBD				
Summers, 2003[279]	*T. suis*	2500TSO	Prednisone, antibiotics, 5-ASA, AZA, 6-Mp	4 CD, 3 UC	open label	CD (CDAI>180) and active UC	+ w8:86% remitted‡	no
Summers, 2005[280]	*T. suis*	2500 TSO every 3w for 24w	14/29 on corticosteroids	29/0	open label	CD (CDAI: 220-450)	+ w12: 75% responded, 65% remitted w24: 79% responded 72% remitted•	no
Summers, 2005[281]	*T. suis*	2500 TSO every 2w for 12w	Mesalamine, corticosteroids/AZA/6-Mp	54/24	randomized, double blind	UC (UCDAI >=4)	12w: 43.3% responded with TSO vs 16.7% placebo ($P = 0.04$)*	no
Elliot, 2005[282]	*T. suis*	2500 TSO every 2w for 12w	Mesalamine, corticosteroids/AZA/6-Mp	?	randomized, double blind, crossover of Summers, 2005	UC (UCDAI >=4)	12w: 56.3% responded with TSO vs 13.3% placebo ($P = 0.02$)*	no
Kradin, 2006[283]	*T. suis*	5x1500 TSO	Refractory to corticosteroids, thalidomide, AZA, and α-TNF	1/0	case report	CD	No benefits	Worms reached adult stage
Broadhurst, 2010[284]	*T. trichiura*	3x eggs (500, 1000, 2000) in 3 years	Refractory to mesalamine 6-MP and steroids.	1/0	case report	Severe UC	Induction of remission	No severe adverse events
Sandborn, 2013[285]	*T. suis*	500, 2500 and 7500 TSO	5-ASA, prednisone, azathioprine, 6-Mp	27/9	sequential dose-escalation randomised, double-blind.	mild-to moderate CD	na	Adverse events in 37% TSO vs 44% placebo patients; no dose dependency
				Multiple sclerosis				
Benzel, 2012[123]; Rosche, 2013[124]	*T. suis*	TSO every 2w over 6 months	2/4 received methylprednisone	4/0	non-randomized, pilot study	secondary progressive	Moderate immune-modulatory effect	1/4 mild gastrointestinal symptoms
Fleming 2011[125]	*T. suis*	TSO every 2w over 3 months	2/5 previously received corticosteroids. Declined standard treatment.	5/0	baseline versus treatment	relapsing remitting ,newly diagnosed	Reduction of new gadolinium-enhancing MRI lesions (n-Gd+).	3/5 mild transient gastrointestinal symptoms
				Allergic rhinitis				
Bager, 2010[111]; Bager 2011[112]; Bourke, 2012[113]	*T. suis*	8x 2500 TSO every 21 days	No use of systemic steroids during the last 2 months and no immune therapy in the last 2 years.	96/47	double-blind, placebo-controlled	na	No therapeutic effect	Moderate to severe gastrointestinal disorders (76% TSO vs 49% placebo). Especially from d0-d42.

TSO: embryonated Trichuris suis ova; 5-ASA: *5-aminosalicylic acid, AZA: Azathioprine*, 6-Mp: 6-Mercaptopurine; CDAI: Crohn's disease activity index; UCDAI: ulcerative colitis disease activity index. na: not assessed.
‡ Remission defined according to the IBD quality of life index. Among the CD patients 3/4 remitted and 4/4 responded (ΔCDAI> 150). All the UC patients responded (SCCAI>4).
* Response defined as ΔUCDAI> -4 point. Calculated according intention to treat.
• Response defined ad ΔCDAI> - 100 points, Remission defined as CDAI < 150

To our knowledge, no other studies of *T. suis* in IBD have been published. In 2010, Brodhurst reported the case of a UC patient who improved its clinical symptoms by self-infecting with multiple doses of *T. trichiura* eggs. Along with the symptomatic remission following the *T. trichiura* treatment, a reduction in neutrophils infiltration and a mucosal regeneration characterized by the presence of $CD4^+$ IL-22^+ Th cells were observed[284]. To date, there is not conclusive evidence of the efficacy of the TSO therapy in IBD and further randomized, double-blind, multicentre studies are needed[286].

1.4.4.1 Concerns on the Safety of Trichuris Therapy

Although *T. suis* infections are presumably transient, self-limiting and do not cause adverse effects in healthy subjects, their outcome in IBD patients is not clear[286]. IBD patients have a compromised barrier function that could favour the invasion of the larvae in the mucosa. Further, the concomitant infection could enhance the inflammatory reaction as observed in *T. muris* infections in colitic mice (Chapter 1.4.3.2). Another major concern is the safety of TSO in immunocompromised host. This is a major concern, since the standard treatment for IBD patients is immunosuppression.

The initial studies performed in IBD patients did not report any adverse effect that could be attributed to the TSO treatment, even in immunosuppressed patients[281]. The larvae reportedly colonised the hosts only transiently for few weeks and no eggs were observed in the faeces of the treated patients, suggesting that the larvae did not reach sexual maturity[279;280;281]. These studies included patients undergoing standard immunosuppressive treatment that did not seem to affect the responsiveness to TSO[281].

A later study, explicitly addressed the concerns on TSO safety with a sequential, dose-escalation, double-blind test on patients suffering from a mild to moderate CD. A similar rate of adverse effects was observed in both TSO and placebo groups and no dose-dependency was noted[285].

Although the initial studies indicate that a *T. suis* infection in humans is not invasive, the parasite might be actually able to invade the intestinal mucosa of the human hosts as it does in the pig. *T. suis* larvae were found in the caecal mucosa of a CD patient treated with TSO. In this case, treatment had no therapeutic efficacy and the mucosa presented a lymphoplasmacytic infiltrate with substantial numbers of eosinophils even in sites not actively infected with *T. suis*[283].

The IBD studies, never found eggs in the faces of the patients, suggesting that *T. suis* never reached sexual maturity[287]. Still a productive infection is possible and was observed once at approximately day 24 in one TSO treated MS patient[115].

The nature of IBD itself might mask the presence of TSO induced symptoms, thereby complicating the assessment of the TSO-treatment related effects. Supporting this concerns, multiple sclerosis (MS) and allergic rhinitis patients developed gastrointestinal symptoms when treated with TSO. In the two

studies with MS patients, 4 out of 9 patients developed transient gastrointestinal effects, that where mild even with concomitant immunosuppression[288;125].

A double-blind, placebo-controlled study confirmed the occurrence of gastrointestinal disorders following TSO administration. Symptoms included moderate to severe flatulence, diarrhoea, and pain in the upper abdomen. The symptoms were transient and occurred particularly up to day 42 post infection[112].

2 OBJECTIVES

2.1 Study Rationale and Aim of the Project

Ulcerative colitis and Crohn's disease frequency increased dramatically in the last 60 years. According to the old friend hypothesis, improved hygienic condition reduced the interaction with organisms that co-evolved with our immune are thus essential for its proper functioning. Several studies have addressed the possibility of reintroducing these missing stimuli to treat inflammatory bowel diseases (IBD) and other disorders.

The whipworm parasite *Trichuris suis* is a promising therapeutic agent, since it colonizes and interacts with the host intestine without causing major pathogenicity. In pigs, the natural host, infection is initiated by the ingestion of embryonated eggs (*Trichuris suis* ova, TSO). In the last two decades, few clinical studies have addressed the safety and efficacy of *T. suis* in IBD and in other immune related diseases. The evidence in support of TSO therapy in IBD is inconclusive. Furthermore, the safety of helminth therapy in immunosuppressed individuals that constitute the majority of the IBD patients is a major concern, and systematic studies are missing.

Basic research is complicated by the absence of an animal IBD model that would allow studying a TSO treatment. In rabbits the course of a TSO infection resembles the one in humans with colonisation of the gastrointestinal tract for 2-3 weeks without reaching sexual maturity.

For this reason we chose to use New Zealand white (NZW) rabbits as an in vivo model to study *T. suis*.

The overall aim of this work was to develop a rabbit model of colitis and use it to evaluate the efficacy and safety of TSO therapy in immunocompetent and immunosuppressed animals.

This work pursued the following specific aims:

1) Establish a rabbit colitis model suitable for the *in vivo* study of *T. suis*
   a. Is it possible to develop a colitis model in rabbits analogous to the DSS colitis in mouse? What are the optimal conditions for induction DSS colitis in rabbits?
   b. What criteria can be identified to monitor and assess the development and severity of the induced colitis?

   ⇨ The colitis model was developed on the basis of the well-established dextran sodium sulphate (DSS) model.

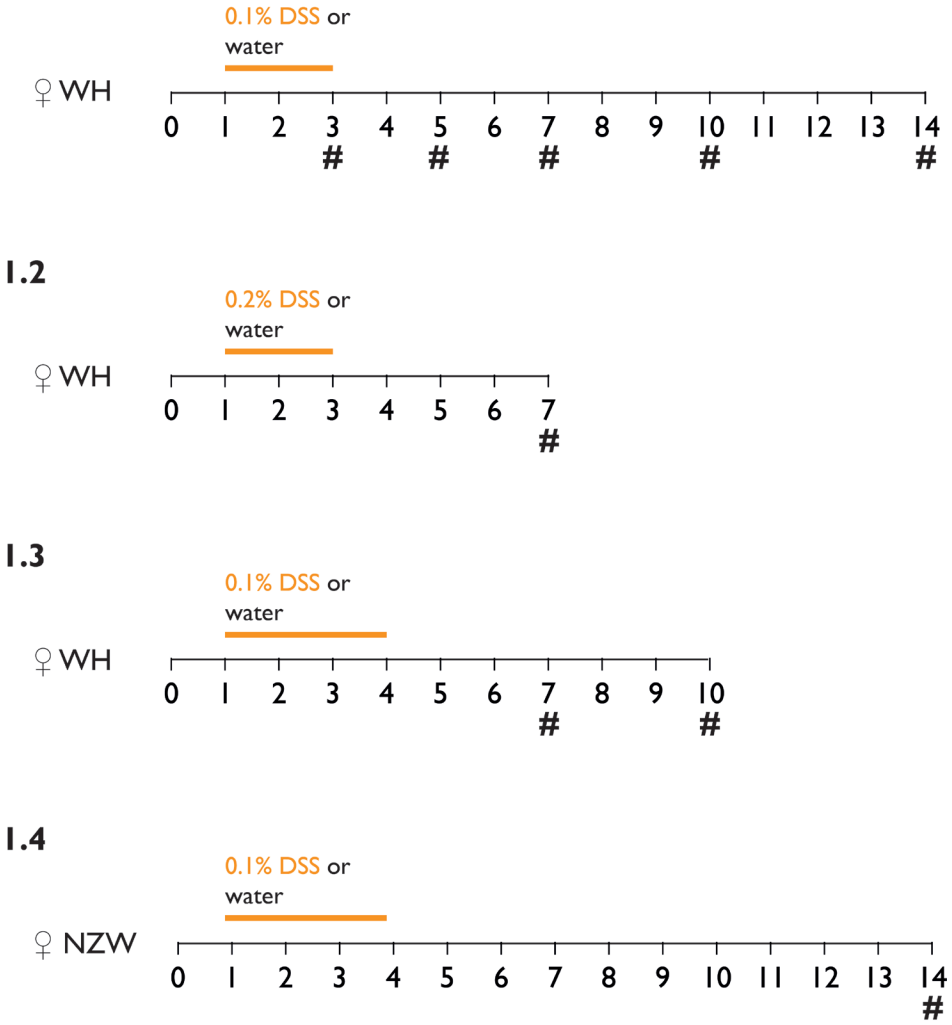

Figure 2.1: Experimental layout for the establishment of a DSS induced colitis suitable for the in vivo study of *T. suis* in the rabbit. Study 1.1, 1.2, 1.3 were performed in White Himalayan rabbits (WH). Study 1.4 was performed in New Zealand White Rabbits. DSS: Dextran Sodium Sulphate, administered in daily beverage; # euthanasia and analysis.

Preliminary studies showed that the dose and dosages used in the murine protocols (3% DSS in drinking water for 10 day) are inadequate for the induction of acute colitis in rabbits as they lead to a fulminant colitis.

We thereby tested different protocols with a considerably reduced DSS concentration and administration length. Figure 2.1 summarises the steps that led do the development of the optimal acute colitis protocol. Clinical symptoms were monitored daily to develop an adequate clinical scoring system. The intestinal tract was examined to identify the sites and the characteristics of the DSS induced pathology. Several biomarkers were analysed

at different time points to identify suitable parameters for the assessment of the colitis severity.

2) Examine the therapeutic effect, the safety, the pharmacodynamics and the mechanisms of action of viable *T. suis* eggs in the colitis model in immunocompetent rabbits

a. What are the optimal doses, dosages and administration of TSO?

b. How do TSO interact with the mucosal immune system and which consequences can be observed on a systemic level?

⇨ We hypothesized that in healthy, immunocompetent rabbits *T. suis* prevents the onset of a chemically induced colitis or reduces its severity.

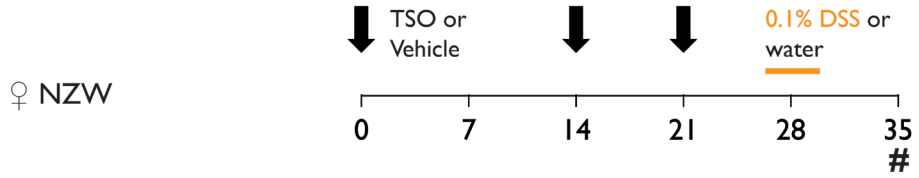

Figure 2.2: Experimental layout for the preventive treatment with TSO ova in a model of DSS induced acute colitis. Studies were performed in New Zealand White Rabbits. DSS: Dextran Sodium Sulphate, administered in daily beverage. TSO: 2500 *T. suis* ova, administered *intra gastrically* ; # euthanasia and analysis.

We tested the effects of TSO administration before the induction of acute colitis. The dose and dosage were chosen on the basis of the protocols utilized in the published human studies (Figure 2.2). The clinical symptoms and colitis severity were monitored on the basis of the previously identified parameters. A transcriptome analysis of immune cells isolated from caecal tissues was performed to investigate how *T. suis* modulates the mucosal niche. We investigated the effects of *T. suis* on the intestinal microbiota by characterizing the microbial composition of the faeces and caecal contents at different time points during the experiments.

3) Examine the therapeutic effect and the safety of viable *T. suis* eggs in the colitis model in immunosuppressed rabbits

a. Which consequences of a TSO therapy can be observed in immunocompromised subjects?

b. Can an immunosuppressed rabbit control a *T. suis* infection?

c. Is a functional immune system a prerequisite for the therapeutic effect of TSO?

⇨ We hypothesized that in immunosuppressed rabbits *T. suis* escapes the control by the immune system and leads to an exacerbated colitis.

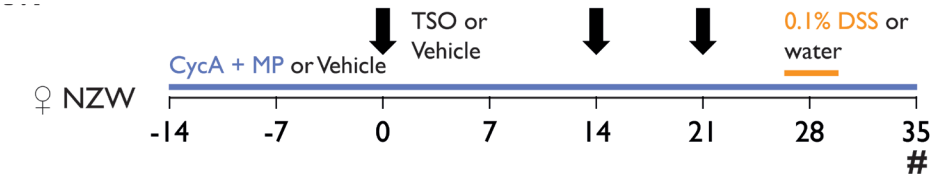

Figure 2.3: Experimental layout for the preventive treatment with TSO ova in a model of DSS induced acute colitis in immunosuppressed rabbits. Studies were performed in New Zealand White Rabbits. Immunosuppression was induced with a combination of Cyclosporine A (CycA) and Methylprednisolone (MP) administered *per oral*. DSS: Dextran Sodium Sulphate, administered in daily beverage. TSO: 2500 *T. suis* ova, administered *intra gastrically;* # euthanasia and analysis.

Cyclosporine and methylprednisolone were used for the establishment of an immune suppression protocol in rabbits. The preventive TSO administration established previously was applied to the immunosuppressed rabbits (Figure 2.3). Samples were collected from different organs to test for a systemic migration of the larvae. The intestinal microbiota of immunosuppressed rabbits was characterized and compared with the composition of immunocompetent rabbits.

2.2 Contribution to Further Projects During the Time of the Dissertation (not addressed in detail within this written thesis)

2.2.1 Protective Role of *Helicobacter pylori* in DSS-Induced Chronic Colitis

Similarly to helminths infections, infections with *H. pylori* have been inversely linked to various allergic and chronic inflammatory conditions. We showed that experimental infection with *H. pylori*, and administration of regular doses of *H. pylori* extract, both alleviate the clinical and histopathological features of DSS-induced chronic colitis.

Publication

Engler DB, **Leonardi I**, Hartung M, Kyburz A, Spath S, Becher B, Rogler G, Müller A.
Helicobacter pylori-specific protection against inflammatory bowel disease requires the NLRP3 inflammasome and IL-18, Inflamm Bowel Dis. 2015 Feb 11. [Epub ahead of print]

Own contribution to the publication:

Euthanasia of mice and sampling
Determination of the murine endoscopic index of colitis severity

2.2.2 Influence of Isotretionin in Chronic DSS-Induced Colitis and T-cell Transfer Colitis

Various studies discuss a potential adverse effect of isotretionin that is used in the treatment of severe forms of acne, in the development of IBD. The role of isotretionin in a chronic DSS-induced colitis and a T-cell transfer colitis model has been assessed.

Publication

Frey-Wagner I, Fischbeck A, Cee A, **Leonardi I**, Gruber S, Becker E, Atrott K, Lang S, Rogler G.
Effects of retinoids in mouse models of colitis: benefit or danger to the gastrointestinal tract? Inflamm Bowel Dis. 2013 Oct;19(11):2356-65.

Own contribution to the publication:

Animal care, treatment, recording of weight curves

2.2.3 pH Receptors in Intestinal Inflammation

A local acidification in the gut lumen as well as in the mucosa occurs during intestinal inflammation and is implicated in the pathogenesis and progression IBD. We investigated whether GPR4, a prototype pH-sensing receptor, is involved in intestinal inflammation. Our data demonstrate that deletion of GPR4 is associated with ameliorated DSS and *Il-10 KO* colitis and thus pH sensing plays an important role in the pathophysiology of IBD.

Publication

Wang Y*, de Valliere C*, **Leonardi I**, Gruber S, Gerstgrasser A, Weber A, Leucht K, Wolfram L, Hausmann M, Krieg C, Thomasson K, Boyman O, Frey-Wagner I, Rogler G, Wagner CA
The proton-activated receptor GPR4 modulates intestinal inflammation
Submitted, 2015, * Shared first authors.

Own contribution to the publication:
LPMC isolation
Design, performance and analysis of the Flow cytometry experiments

2.2.4 Role of Transcriptional Factor Nfr2 in Mucosal Inflammation and Cancer

The transcription factor Nrf2 is a major modulator of the cellular antioxidative response. In IBD the regulation of reactive oxygen species (ROS) is of high interest, as the mucosa of patients is infiltrated by macrophages leading to a massive production of ROS. Transgenic mice conditionally expressing a constitutively active form of *Nrf2* (ca*Nrf2*) were used to study the effects of *Nrf2* overexpression in acute DSS colitis and chronic *Il-10 KO* colitis. Mice overexpressing *Nrf2* in epithelial cells or myeloid cells lost more weight during acute colitis, had a more pronounced shortening of the colon and an elevated histological score. The worsening of inflammation in an acute model could not be confirmed in a chronic *Il-10 KO* model, where even a tendency towards reduced prolapse rate in female mice was observed.

Publication
Cee A, **Leonardi I**, Atrott K, Kopf M, Schäfer M, Werner S, Rogler G, Frey-Wagner I
Cell-specific activation of the Nrf2 antioxidant pathway increases mucosal inflammation in acute but not in chronic colitis. Submitted, 2015.

Own contribution to the publication:
Euthanasia of mice and sampling
Determination of the murine endoscopic index of colitis severity
Histological scoring
Isolation of murine IECs

3 FIRST MANUSCRIPT

Oral Administration of Dextran Sodium Sulphate Induces a Caecum Localized Colitis in Rabbits

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The authors apologize for this error.

Published in: **IntJExpPath,** 2015 Feb 26, doi: 10.1111/iep.12117

Own contribution to the publication:

Animal care, IS treatment, recording of weight curves (IL)

Euthanasia of rabbits, samples collection (IL, FN)

Biochemical assays (IL)

Histological scoring (IL, AC)

Isolation of rabbit LPMC, IECs (IL)

Quantitative rtPCR (IL)

Analysis of transcriptome data (IL)

Study design (IL,FN, IFW, GR,)

Manuscript writing (IL, IFW, GR)

Figure design and arrangement (IL)

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Zurich, Juli 01, 2015

Dear Editors

In our manuscript Leonardi et al, "Oral administration of dextran sodium sulphate induces a caecum-localized colitis in rabbits" Figure 6 was printed twice and the actual Figure 7 is missing.
The correct figure 7, along with the accompanying figure legend, is:

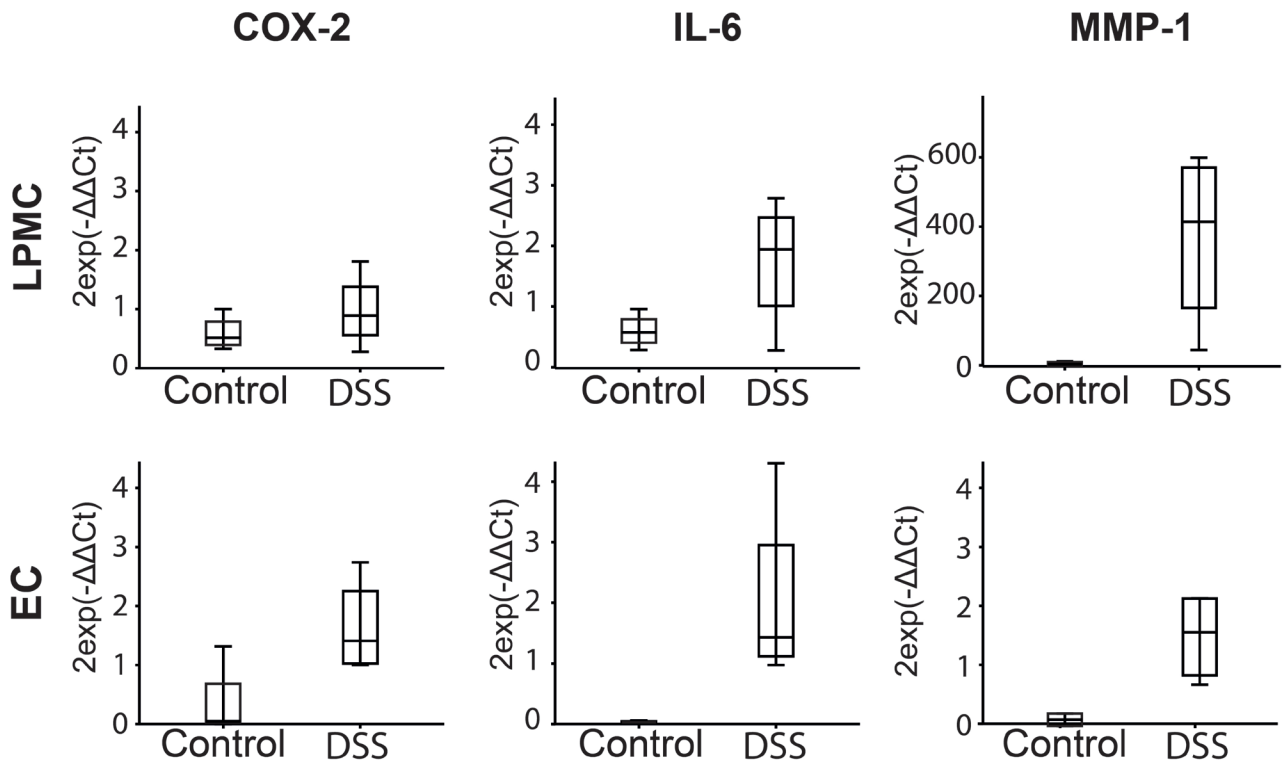

Figure 7: Quantitative RT-PCR showing expression of COX-2 (a, d), IL-6 (b, e) and MMP-1 (c, f) in intestinal epithelial cells (IEC, upper panel) and lamina propria mononuclear cells (LPMC, lower panel) of DSS and control rabbits at day 10 post colitis induction. Expression is shown relative to GAPDH in the distal colon, *n* = 4–9. Values are given as mean ± SD and difference between groups was tested by two-tailed Student's *t*-test.

We apologize for this error.

Yours sincerely,

Gerhard Rogler

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ORIGINAL ARTICLE

Oral administration of dextran sodium sulphate induces a caecum-localized colitis in rabbits

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SUMMARY

Trichuris suis ova (TSO) have shown promising results in the treatment of inflammatory bowel disease (IBD) but the mechanisms which underlies this therapeutic effect cannot be studied in mice and rats as *T. suis* fails to colonize the rodent intestine, whilst hatching in humans and rabbits. As a suitable rabbit IBD model is currently not available, we developed a rabbit colitis model by administration of dextran sodium sulphate (DSS). White Himalayan rabbits (n = 12) received 0.1% DSS in the daily water supply for five days. Clinical symptoms were monitored daily, and rabbits were sacrificed at different time points. A genomewide expression analysis was performed with RNA isolated from caecal lamina propria mononuclear cells (LPMC) and intestinal epithelial cells (IEC). The disease activity index of DSS rabbits increased up to 2.1 ± 0.4 (n = 6) at day 10 (controls <0.5). DSS induced a caecum-localized pathology with crypt architectural distortion, stunted villous surface and inflammatory infiltrate in the lamina propria. The histopathology score reached a peak of 14.2 ± 4.9 (n = 4) at day 10 (controls 7.7 ± 0.9, n = 5). Expression profiling revealed an enrichment of IBD-related genes in both LPMC and IEC. Innate inflammatory response, Th17 signalling and chemotaxis were among the pathways affected significantly. We describe a reproducible and reliable rabbit model of DSS colitis. Localization of the inflammation in the caecum and its similarities to IBD make this model particularly suitable to study TSO therapy *in vivo*.

Keywords
Crohn's disease, DSS colitis, rabbit model of inflammatory bowel disease, RNA sequencing, *Trichuris suis*, ulcerative colitis

Inflammatory bowel diseases (IBD) can be regarded as a 'postindustrial revolution epidemic' as the frequency of these diseases has increased dramatically in the last 60 years (Molodecky *et al.* 2012). Initially, this increase was explained on the basis of the hygiene hypothesis that linked the improved hygienic conditions and the consequent reduction of childhood infections to an increase in the prevalence and incidence of immune-related diseases (Strachan 1989). Today it is assumed that the increase in hygiene standards reduces the interactions with micro-organisms that co-evolved with the immune system and influences the balance between immune-regulatory and effector mechanisms (Rook 2011).

In 2000, Elliot and colleagues focused their attention on the complementarity between the distribution of IBD and of helminth infections (Elliott *et al.* 2000; Weinstock *et al.* 2002). In their work they set the basis for the clinical application of a helminth therapy and proposed the whipworm parasite *Trichuris suis* as a therapeutic agent (Summers *et al.* 2003). Overall, the treatment with *T. suis* ova (TSO) proved to be safe with only mild and transient gastrointestinal effects reported (Scholmerich 2013). Those early results for efficacy in IBD were promising. However, two recent large multicenter trials in mild to moderate Crohn's disease with or without immunosuppression could not demonstrate a significant benefit of TSO treatment over placebo. Further clinical trials in ulcerative colitis are still under discussion. So far most studies have focused on the clinical efficacy and safety, and the mechanisms underlying the TSO treatment

effects remain unsolved. The discussion on whether further clinical studies should be undertaken (i.e. in ulcerative colitis) has given rise to a request for a better understanding of potential mechanisms and this implies establishment of suitable animal models.

Animal models of colitis are essential for the understanding of the aetiology and pathophysiology of IBD and constitute an essential tool in the development of new therapies. Currently, more than 66 different IBD models have been developed in several species including mouse, rat, rabbit and tamarin (Wirtz & Neurath 2000). Generally, IBD models can be subdivided into four categories of experimental colitis depending on the method of induction: congenital, genetically engineered, chemically induced and cell transfer induced (Mizoguchi 2012).

Most methods are used successfully in both mouse and rat. Unfortunately, research on the therapeutic application of *T. suis* in model organisms is complicated by the unsuccessful hatching of the ova in the mouse and rat intestine (unpublished data). In contrast, the life cycle of these parasites in the human and rabbit intestine is similar. In both hosts, *T. suis* hatch and establish in the distal intestine region where the larvae seem to die prematurely without reaching sexual maturity (unpublished data). Therefore, a colitis model in the rabbit would be a valuable tool to investigate the mechanisms underlying *T. suis* therapy.

Thus far the established rabbit IBD models have several drawbacks that limit their use for translational research. Rectal application of acetic acid causes severe acute inflammation, ischaemia and erosion within one day postapplication, but fails to induce chronic inflammation (Hathaway *et al.* 1999; Murthy 2006). Similarly, trinitrobenzene sulphonic acid (TNBS) dissolved in ethanol is also applied in the rectum. Within a week after application, TNBS induces a fully developed inflammation that presents ulcerative lesions and transmural inflammation (Anthony *et al.* 1995, 2007). However, the development of chronic inflammatory lesions in the TNBS model is highly variable and does not show good reproducibility (Knollmann *et al.* 2002). Furthermore, the short-term and self-limiting nature of these colitis models is not adequate for the study of the relapsing and remitting course of IBD. Both acetic acid and TNBS are introduced as an enema in the rabbit rectum and induce an inflammation that is usually confined to the distal colon, whereas *T. suis* ova hatch and develop in the ileum and caecum. Further colitis models in rabbits are of limited use either because of the complicated induction procedure (Hodgson *et al.* 1978; Hotta *et al.* 1986) or because of the high variability of the induced pathology (Watt & Marcus 1970).

Thus, a novel colitis model in the rabbit that allows the study of the mechanisms underlying the therapeutic effects of TSO treatment is needed.

In both mice and rats experimental colitis is commonly induced by the heparin-like polysaccharide dextran sodium sulphate (DSS). DSS increases the trans-epithelial permeability by decreasing the expression and by inducing the redistribution of tight junction proteins (occludin, zonula occludens-1, claudins) and by enhancing epithelial cell apoptosis (Poritz *et al.* 2007), (Yan *et al.* 2009), (Mennigen *et al.* 2009). Furthermore, DSS causes a hyperosmotic stimulus that leads to the activation of NF-κB in the epithelium (Schwartz *et al.* 2008). This is consistent with the accepted role of epithelial barrier dysfunction in the pathogenesis of IBD (Clayburgh *et al.* 2004). In both IBD and DSS colitis, the damaged epithelium allows the entry of luminal content into the mucosa, thereby facilitating the onset of inflammatory processes (Nell *et al.* 2010). In mice short-term administration of DSS (1–10% w/v) in drinking water is used to induce 'acute' colitis, whereas long-term or cyclic administration produces chronic colitis (Wirtz *et al.* 2007). The development of pathology can be easily monitored based on body weight, stool appearance, rectal bleeding and behavioural changes. Such clinical changes are usually preceded by alterations in histopathological parameters including colon shortening, mucosal injury, immune infiltration and epithelial damage. These changes initially appear in focal regions of the distal colonic mucosa and then expand progressively although the inflammation remains confined to the colon (Melgar *et al.* 2005).

The DSS model guarantees low risk of mortality, high reproducibility and good uniformity of the induced mucosal inflammation (Melgar *et al.* 2008). We therefore chose to develop a DSS colitis model in the rabbit. We found that in rabbits administration of 0.1% DSS for 5 days induces a clear caecum-localized inflammation that mimics histological features of ulcerative colitis and is characterized by a similar gene expression profile as observed in biopsies from patients with IBD. Furthermore, we describe a scoring system to correlate clinical parameters with histopathological findings that should facilitate the evaluation of the tested therapeutic approach.

Methods

Rabbits

All animal experiments were carried out according to Swiss animal welfare laws and approved by the veterinary office of Zurich. Female white Himalayan rabbits and New Zealand white rabbits (Charles River, Kisslegg, Germany) weighing 1.9–2.1 kg were used for the experiments. Rabbits were maintained single-housed with water and food (standard rabbit maintenance diet – Provimi Kliba AG, CH-4303 Kaiseraugst, hay and straw) *ad libitum* on a 12:12 h light/dark cycle. Upon arrival, animals were kept for at least four days under routine husbandry. One week prior to DSS exposure, drinking water was substituted by organic fennel tea (Hipp, Pfaffenhofen, Germany) *ad libitum*.

Colitis induction and clinical evaluation

Colitis was induced by DSS (MP Biomedicals, Illkirch, France) dissolved in cold fennel tea at 0.1% w/v (if not specified otherwise). Control rabbits received fennel tea as vehicle. The beverage was prepared freshly and changed at least every second day. For every animal daily weight, daily food and fluid intake, daily stool appearance and behaviour were monitored. A disease activity index was calculated according to Table 1. The disease activity (range: 0–4) index represents the sum of individual scores for weight loss, presence of uneaten cecotrophs, food intake and beverage intake divided by 4. Euthanasia was performed following sedation with barbiturates with an overdose of ketamine hydrochloride (Vétoquinol, Bern, Switzerland) and xylazine (Bayer, Lyssach, Switzerland).

The abdominal cavity was exposed by a midline laparotomy, and samples were collected from the ileum, jejunum, duodenum, caecum and colon. For RNA extraction and myeloperoxidase activity analysis, the excised samples (0.5 cm in length) from the duodenum, jejunum, ileum and colon were opened by a longitudinal incision and rinsed with cold PBS. 1 cm^2 sections of the caecum were washed extensively with cold PBS until removal of the luminal content was complete. The samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. For histological analysis samples (0.5 cm^2 sections of the caecum samples or 0.5 cm length sections of the other tissues) were either cut longitudinally or cut into smaller (0.2 cm) sections for fixation. The samples were carefully washed and fixed with phosphate-buffered 10% formalin solution. For genomewide mRNA expression studies, caecal samples (2 cm^2) were washed extensively with cold PBS and stored on ice in 5% BSA in PBS until further processing.

Whole caecal tissue RNA extraction and quantitative real-time RT-PCR (qPCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and the automated sample preparation system Qiacube (Qiagen) following the manufacturer's recommendations. cDNA was synthesized with the High-capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, California, USA).

To study the transcription of immune response markers in rabbit intestine tissues, sequence-specific primers were applied (Table 2) and qPCR was performed according to Schnupf *et al.* with some modifications (Schnupf & Sansonetti 2012). Amplifications were performed in a total volume of 15 µl including 50 ng of cDNA, primers (0.2 µM each) and 7.5 µl of Power SYBR Green mix (Life Technologies). Reactions were run in triplicate on an ABI 7900HT (Life Technologies) using the universal thermal cycling parameters (2 min 54°C, 94.5°C 10 min, 40 cycles of 15 s at 97°C and 60 s at 59.7°C; dissociation curve: 15 s at 95°C, 15 s at 60°C and 15 s at 95°C). Results were analysed with the sequence detection software ABI 7900HT SDS2.4. For quality control purposes, all samples' dissociation curves were acquired and amplification products were visualized by 2% agarose gel electrophoresis. Primer sequences are listed in Table 2. The comparative $\Delta\Delta C_t$ method was applied for relative gene expression quantification (C_t: threshold cycle).

Isolation of caecal lamina propria mononuclear cells (LPMC) and intestinal epithelial cells (IEC)

Caecal LPMC and IEC were isolated as previously described (Weigmann *et al.* 2007), with some

Table 1 Scoring system for the daily monitoring of the disease activity index

Score	Weight loss	Stool appearance and cecotrophs	Reduction in food intake	Reduction in beverage intake	Fur appearance
0	None	Well-formed solid pellets, 0 cecotrophs	None	None	Clean, bright fur
1	0–2%	Easy to smear and loose stool, ≤1 cecotrophs	0–30%	0–30%	Dim fur
2	2–5%	Loose stool, 2–3 cecotrophs	30–60%	30–60%	Shagged fur
3	5–10%	Loose smeared stool in cage, 4–5 cecotrophs	60–90%	60–90%	Smudgy, unclean fur
4	>10%	Loose smeared stool in cage, > 5 cecotrophs	>90%	>90%	Smudgy, stool stains, smeared anus

Table 2 Primers for qPCR

Marker	Forward primer Backward primer	Primer location within CDS	Target size	NCBI Accession
IL-12p35	AAGGCCAGACAAACTCTAGAATTC TTGGTTAACTCCAGTGGTAAACAGG	Exon 3/4 and 4/5 from ~8	116 nts	XM_002716291
iNOS	GACGTCCAGCGCTACAATATCC GATCTCTGTGACGGCCTGATCT	Undetermined	102 nts	XM_002718780
IFNγ	TGCCAGGACACACTAACCAGAG TGTCACTCTCCTCTTTCCAATTCC	Exon 1 and 2/3 from 4	127 nts	NM_001081991
GAPDH	TGACGACATCAAGAAGGTGGTG GAAGGTGGAGGAGTGGGTGTC	Exon 1 of 1	120 nts	NM_001082253

modifications. Briefly, the dissected mucosa was washed with Ca^{+}- and Mg^{+}-free PBS; the caecal fold was removed and discarded. The tissue was cut and incubated in medium containing 20 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C on a shaking platform (150 rpm). After incubation, the suspension of IEC, villus cells, subepithelial cells and intestinal epithelial lymphocytes was detached by vortexing and passing through a 70-μm cell strainer (BD Biosciences, Erembodegem, Belgium). The epithelial cells were washed twice, pelleted, resuspended in RTL buffer (Qiagen), snap-frozen in liquid nitrogen and stored at −80°C for later analysis. The remaining tissue containing LP with muscle layer was collected and incubated in medium containing 1 μg/ml collagenase type I CLS (Worthington Biochemical Corp., Freehold, New Jersey, USA) at 37°C on a shaking plate (300 rpm). After 15 min incubation, the suspension was vortexed and filtered through a 70-μl strainer. The filtered cells were resuspended in 5% BSA in PBS to stop the enzymatic digestion. The undigested tissue was incubated with fresh collagenase solution for additional 15 min. The collagenase digestion was repeated three times, and the washed LPMC were pooled. LPC were pelleted twice and resuspended in DMEM supplemented with 5% FCS. LPMC were purified using Ficoll-Paque PLUS (GE Healthcare Europe GmbH, Freiburg Germany) gradient centrifugation for 40 min at 150×g. The viability of the cells was confirmed by tryptan blue staining. Cells were resuspended in RTL buffer (Qiagen), snap-frozen in liquid nitrogen and stored at −80°C for later analysis.

RNA isolation and genomewide mRNA expression analysis

Total RNA was isolated with the Qiacube system using the RNeasy Mini Kit with DNase digestion (Qiagen) to eliminate genomic DNA. RNA integrity and quantity were determined on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Samples with an integrity score ≥6.8 were sent to the Functional Genomic Centre Zurich (FGCZ) for sequencing on the Illumina® platform. The fold change (FC) was used to express the changes in average gene expression between studied groups. FC was normalized against the control group (rabbits receiving fennel tea only). The ENSEMBLE IDs were annotated using BetterBunny augmented annotation and analysis of rabbit genes (http://cptweb.cpt.wayne.edu) (Craig *et al.* 2012). MetaCore™ (Thomson Reuters, http://portal.genego.com) was used to perform network and pathway analyses. The following cut-offs were applied to select differentially expressed genes for further analysis: *P*-value $P \leq 0.01$ and fold change ≥|2.0|. The pathways (groups of genes belonging to the same pathway map in MetaCore™ database) and gene families were considered significant with a *P*-value ≤0.05 and were further selected on the basis of their relevance to inflammatory bowel disease pathology. Additional gene expression data sets for comparison were obtained from GEO Data Sets (NCBI) of previously published studies in colon pinch biopsies from patients with UC and CD (Granlund *et al.* 2013).

Validation of the genomewide mRNA expression analysis

The expression profiling results were confirmed by qPCR of selected genes involved in the highlighted pathways. cDNA synthesis was performed using a High-capacity cDNA Reverse Transcription Kit (Life Technologies Ltd). Real-time PCR was performed using TaqMan Gene Expression Assays (Life Technologies Ltd) and TaqMan Fast Universal PCR Master Mix No AmpErase UNG (Life Technologies Ltd) on a 7900 HT Fast Real-Time PCR System with SDS 2.2 Software (Life Technologies Ltd). TaqMan gene expression assays were performed for COX-2 (Ptgs2, Oc03398293 _m1), IL-6 (Oc04097051_m1), MMP-1 (Oc04250656_m1)

Table 3 Scoring system for DSS-induced histological changes in the caecum

	Morphological features			Inflammation		
	Villous stunting	Villous epithelial injury	Crypt distortion	Intraepithelial lymphocytes	LP lymphocytes and plasma cells	LP eosinophils
1	Normal mucosa	Normal mucosa	Normal mucosa	5–10/50 IEL/epithelial cells	25% of the villous lamina propria	2–3 cells per × 40 field
2	Mild villous stunting	Mild villous epithelial injury	Mild crypt distension, hyperplasia and distortion	11–30 IEL/50 epithelial cells.	25–50% of the villous lamina propria	5–10 per × 40 field.
3	Moderate villous stunting	Moderate villous epithelial injury	Moderate crypt distension, hyperplasia and distortion.	31–50 IEL/50 epithelial cells may be focally clustered.	50–75% of the villous lamina propria.	10–20 per × 40 field.
4	Marked villous stunting	Marked villous epithelial injury	Marked crypt distension, hyperplasia and distortion	51–100 IEL/50 epithelial cells, may be clustered and at all levels of the epithelium	75–100% of the villous lamina propria.	> 20 per × 40 field

and the housekeeping gene GAPDH (Oc03823402_g1) as an endogenous control. Measurements were performed in triplicates; relative expression was calculated using the ΔΔCt method.

Histopathological evaluation of colitis

After careful dissection and fixation, tissues were routinely embedded in paraffin. Serial sections of 5 μm were cut using a microtome (Carl Zeiss AG, Feldbach, Switzerland) and stained with haematoxylin–eosin to investigate epithelial damage and cellular infiltration. The histological changes in the caecum were quantified in a blinded manner by two investigators with a scoring system (range 1–24) for morphological features and infiltration of immune cells according to the scoring system described in Table 3 (Cooper *et al.* 1993; Kojouharoff *et al.* 1997; Day *et al.* 2008).

Analysis of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured in different regions of the gastrointestinal tract as previously described. Myeloperoxidase activity was calculated as mean absorbance (460 nm) per incubation time per protein content of

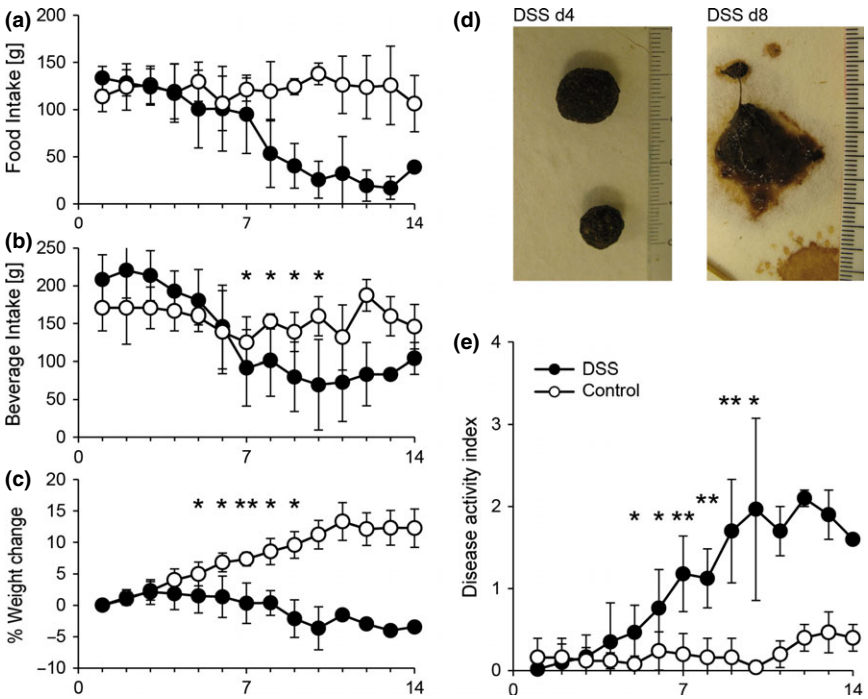

Figure 1 Manifestation of clinical symptoms upon DSS exposure. Response to colitis induction was monitored daily according to a detailed score sheet. Food intake (a), beverage intake (b), weight change (c), stool consistency (d. representative pictures for control and DSS rabbits at day 10) were summarized into a disease activity index (e. DAI, 0–4). DSS rabbits (●, $n = 12$) were fed with 0.1% DSS in the daily beverage (fennel tea) for 5 days. The control group (○, $n = 5$) was maintained under the same conditions with fennel tea as beverage. Data represent mean ± SD; Mann–Whitney test, $^{**}P \leq 0.005$, $^{*}P \leq 0.05$.

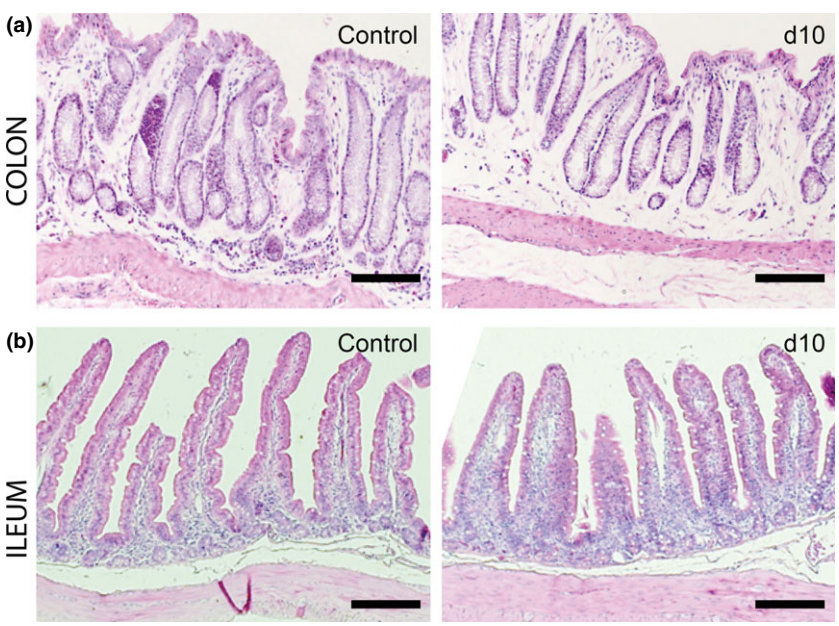

Figure 2 Absence of histopathology in the colon and ileum. Representative HE-stained colon (a) and ileum (b) sections in control and in DSS-exposed rabbits at day 10. Scale: 200 μm.

the sample in grams (indicated as arbitrary units U/g s) (Bozeman *et al.* 1990).

Statistical analysis

The results of the 0.1% DSS colitis were obtained in two different experiments ($n = 8$ and $n = 4$). As the experimental protocol was identical for both experiments, results were pooled together. The data obtained from this study were analysed using IBM SPSS statistic 21 (Armonk, NY, USA). The majority of the examined parameters were asymmetrically distributed. For the comparison of the treatment groups, the nonparametric Mann–Whitney *U*-test for two independent samples was used.

Results

Clinical symptoms of DSS exposure in rabbits

As rabbits have a more sensitive digestive tract in comparison with mice, the concentration of DSS to induce colitis had to be drastically decreased. We observed a reduction of daily fluid intake that we ascribed to the unpleasant taste of DSS. To overcome this problem, DSS was dissolved in organic fennel tea that successfully masked the taste of the DSS and restored a normal fluid intake during DSS exposure.

A pilot study (data not shown) showed that administration of 0.1% DSS in fennel tea for 3 days reduced the

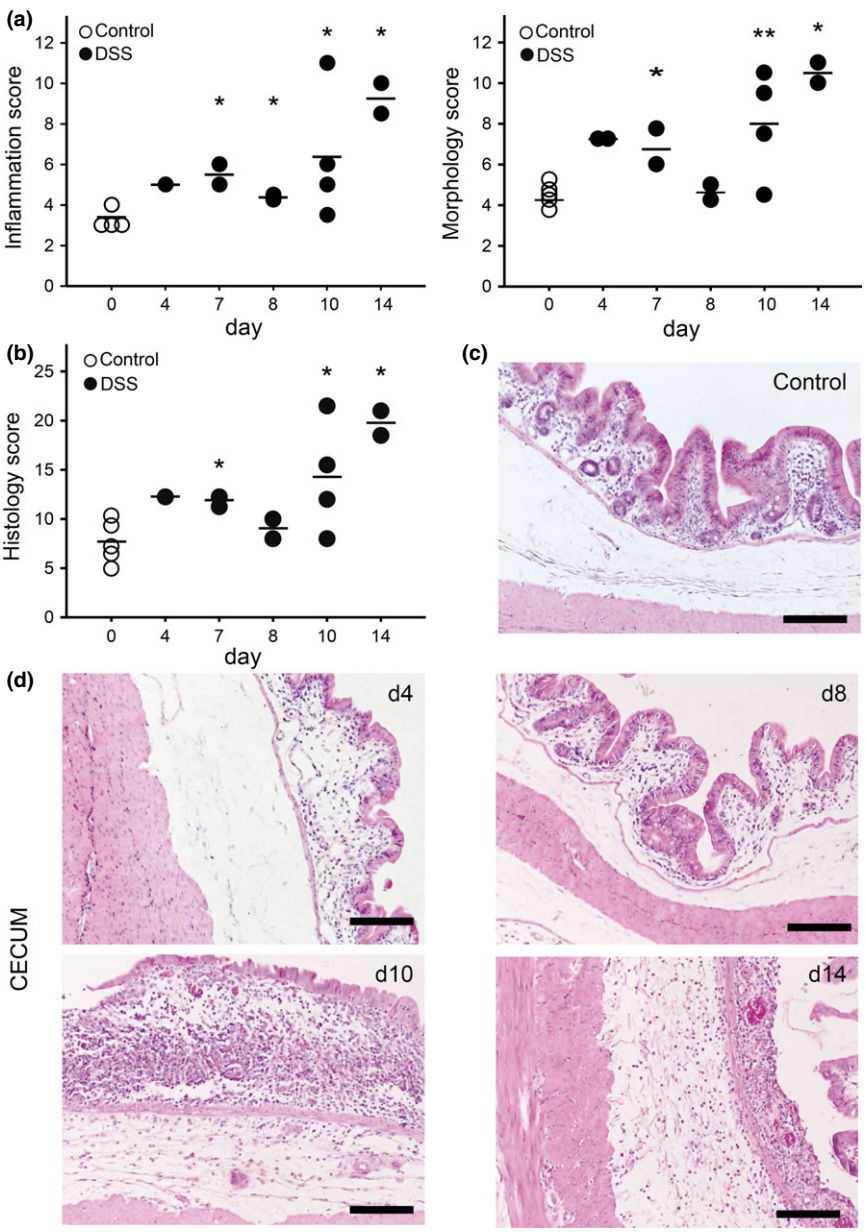

Figure 3 Histopathological changes of the caecum at different time points after colitis induction. HE-stained caecum sections were scored (a, 1–4) for markers of inflammation (infiltration of lamina propria eosinophils, lamina propria lymphocytes, intraepithelial lymphocytes) and for the distortion of morphological features (villous stunting, villous epithelial injury, crypt distortion). Single parameters were summarized to a global score (b). Black dots represent DSS-treated rabbits (●); white dots represent control rabbits (○). Horizontal lines represent the arithmetical mean; Mann–Whitney test, $^{**}P \leq 0.05$, $^{*}P \leq 0.1$. Representative HE-stained caecal sections of control rabbits (c) and of DSS-exposed rabbits at different time points after colitis induction (d). Scale: 200 μm.

normal weight gain from day 5 on in the treated animals. This effect was no longer present from day 14 on, indicating a restitution of the colitis. Other clinical symptoms were not evident. Histological analysis of HE stained intestinal samples showed no clear signs of inflammation; only a slight reduction in the number of goblet cells in the caecum at day 7 and 10 was observed. Subsequently, the duration of the DSS phase was increased from 3 to 5 days. Twelve white Himalayan rabbits were fed for 5 days with 0.1% DSS in the daily beverage (fennel tea), whilst control rabbits ($n = 5$) housed in the same facility were given fennel tea without DSS. The earliest symptoms manifested at day 4, when the rabbits started to gradually diminish the daily food and beverage intake from the initial 120 g/day pellet and 210 ml/day beverage intake at day 1 down to 40 g/day and 100 ml/day at day 7 (Figure 1a, b).

Exposure to 0.1% DSS markedly reduced the weight gain (Figure 1c). Further symptoms included the presence of loose and smeared stool (Figure 1d), behavioural abnormalities such as apathy or aggressiveness and unclean fur. A combinatorial index of disease, (Figure 1e, disease activity index DAI, described in the methods section) was used to quantify the severity of the monitored clinical symptoms. We found that whilst the DAI of control rabbits remained

Table 4 Genes concordantly upregulated in LPMC of DSS colitis rabbits and IBD biopsies

Cell adhesion and cytoskeleton reorganization	Metabolism and biosynthesis
CD38	PTGS2(COX2)
PLEK	SLC6A14[a]
S100A9	TCN1
SELL[a]	
Cytokine and cytokine R genes	Development
IL1A	EGR2[a]
IL6	
IL8	
IRF1	
Chemokine and chemokine R genes	Tissue remodelling genes
CCR7[a]	MMP3
CXCL10	
CXCL11	
CXCR4	
ENA-78[a]	
Immune response	
Innate immune defence	*BCR and TCR signalling*
TMEM173	CD19
DMBT1	LAX1
FAM65B[a]	
SLC11A1[a]	

List of genes concordantly upregulated in LPMC from the caecum of DSS-treated rabbit and colonic biopsies of inflamed tissue from patients with IBD. LPMC: Lamina propria mononuclear cells. [a]differentially expressed in patients with UC, only.

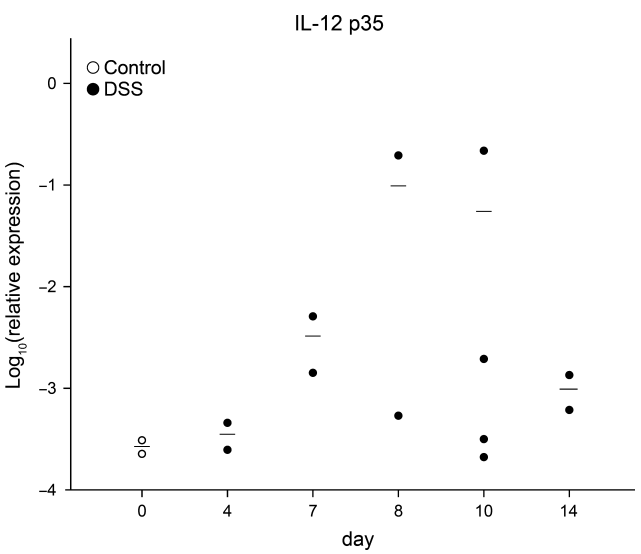

Figure 4 mRNA expression of pro-inflammatory cytokines during colitis induction. mRNA expression of IL-12 p35 in caecum from DSS-treated (●) or control (○) rabbits. Results are shown as mean expression relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method. Dots represent single rabbits; horizontal lines represent the arithmetical mean. Each sample was analysed in triplicate.

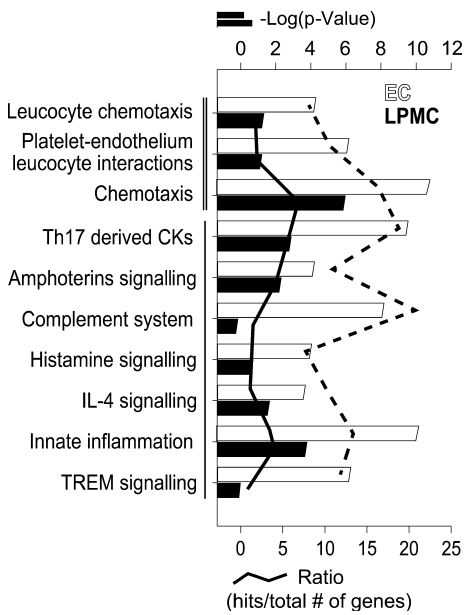

Figure 5 Process network analysis of differentially expressed genes in epithelial cells (EC, black bars and line) and lamina propria mononuclear cells (LPMC, grey bars and line). This analysis is based on a manually curated database of process networks, which details more specific biological processes than GO annotations alone. Most prominent process networks associated with the identified genes were involved in cell adhesion and chemotaxis (═) and immune and immune responses (▬). Analysis was performed with MetaCore™. Bars represent the log(p-value) of enriched pathways, whereas lines represent the ratio between differentially expressed genes upon DSS exposure and the total number of genes involved in the specific process network. Gene expression threshold: fold change $\geq |2.0|$; P-value ≤ 0.05.

at baseline (DAI <0.5), the DAI of rabbits receiving DSS increased significantly starting from day 5 (DSS: 0.47 ± 0.32, $n = 12$; control: 0.08 ± 0.10. $n = 5$) and rose up to 2.1 at day 12.

DSS induces a caecum-localized pathology

Histological evaluation of the intestinal tract revealed a caecum-localized pathology, whereas no clear signs of tissue damage were observed in other regions of the intestinal tract (colon and ileum, Figure 2).

Histopathology of the caecum was characterized by infiltration of immune cells into the epithelial layer and the lamina propria and by morphological changes such as villous stunting, crypt distortion and villous epithelial injury (Figure 3a,c,d). The global histology score was increased from day 4 onward (Figure 3b). The severity of the damage increased progressively even after the removal of DSS from a baseline value of 7.7 ± 0.9 in control rabbits ($n = 5$) to an average score of 13.3 ± 5.0 at day 10 ($n = 4$) and further increased until day 14.

Expression analysis of genes involved in the immune response

To evaluate the inflammatory response in DSS-treated rabbits, we analysed the mRNA expression of inflammation-related genes by q-rtPCR. iNOS, IFNγ and IL-12 p35 were chosen as they have been implicated in IBD and have been shown to be overexpressed in inflamed rabbit tissue (Schnupf & Sansonetti 2012). Gene expression analysis of inflammatory markers in the caecum showed a transient increase of IL-12 p35 in DSS-treated rabbits (Figure 4), although, owing to the limited sample number in the control group, the difference did not reach statistical significance. No difference between the groups was observed for iNOS and IFNγ.

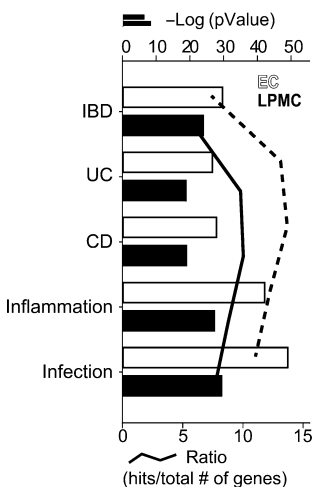

Figure 6 Enrichment of differentially expressed genes in selected disease categories (by biomarkers) in epithelial cells (IEC, white bars and line) and lamina propria mononuclear cells (LPMC, grey bars and line). The Gene IDs of the orthologous genes assigned to the differentially expressed rabbit mRNAs were analysed for enrichment in selected disease categories using MetaCore™. Terms relevant for IBD and experimental colitis are displayed. Bars represent the log (*P*-values) of enriched pathways, whereas lines represent the ratio between the differentially regulated genes upon DSS exposure and the total number of genes involved in the specific process network. IBD, inflammatory bowel disease, UC, ulcerative colitis, CD, Crohn's disease. Gene expression threshold: fold change ≥ |2.0|; *P*-value ≤0.05.

Genomewide gene expression analysis in LPMC and IEC by RNAseq

Genomewide gene expression analysis identified 470 differentially expressed genes in IEC and 215 differentially expressed genes in LPMC (FC ≥ |2|, $P \leq 0.05$). The process networks that were significantly over-represented in

Table 5 Genes concordantly upregulated in ECs of DSS colitis rabbits and IBD biopsies

Cell adhesion and cytoskeleton reorganization	Metabolism and biosynthesis
SELL[a]	FCRLA[b]
PLEK	PLA2G7[a]
VNN1	SLC11A1[a]
S100A9	SLC2A3
CLEC4A	SLC6A14[a]
CD38	TCN1
Cytokine and cytokine R genes	Apoptosis
IL1A	UBD
IL1B	IER3[a]
IL6[a]	PEA15[a]
IL8	
Chemokine and chemokine R genes	Cell–cell signalling
CCR7[a]	ADM
CXCL10	TNFAIP6[a]
CXCL11	**Tissue remodelling genes**
CXCL13	CTSK
CXCL5[a]	MMP1
CXCL6	MMP12
CXCL9	SERPINE2
Immune response	
Innate immune defence	*BCR and TCR signalling*
OAS2	CD19
TLR8[b]	CD74
Humoral immune response	CD79B
POU2AF1	CD86[a]
CD83[a]	LYN[a]
Acute-phase response	SLAMF8[a]
SERPINA1	*Inflammatory response*
Antigen processing	NFKBIZ[a]
HLA-DMA	*Anti-inflammatory response*
HLA-DPA1	A1F1
HLA-DPB1[b]	

List of genes concordantly upregulated in EC from the caecum of DSS-treated rabbit and colonic biopsies of inflamed tissue from patients with IBD. EC: epithelial cells. [a]genes differentially expressed in patients with UC, only. [b]genes differentially expressed in patients with CD, only.

the MetaCore™ analysis were 58 in LMPC and 49 in IEC respectively. Among the most relevant process networks, there was an over-representation of genes involved in inflammation, immune response and chemotaxis (Figure 5).

Furthermore, the disease (by biomarkers) ontology in MetaCore™ was used to assess the similarity between the gene expression in our rabbit DSS colitis model with the gene expression known to be associated with selected human diseases. In both LPMC and EC, we found an enrichment of differentially expressed genes associated with inflammation, IBD, CD and UC (Figure 6).

To further confirm the results of the disease enrichment analysis, we compared genes differentially regulated in our DSS colitis model with a gene set from a genomewide gene expression analysis in human CD and UC patients (Granlund *et al.* 2013). Overall, the majority of the differentially expressed genes were involved in immune response, cell adhesion, cytoskeleton reorganization and chemokine signalling (Table 4; Table 5).

Finally, the sequencing results for mRNA expression were validated for COX-2, IL-6 and MMP1 by qPCR (Figure 7). In accordance with our transcriptome results, the expression of the selected genes was higher in both LPMC and EC isolated from DSS-treated rabbits in comparison with the non-colitic controls.

DSS transiently increases neutrophil infiltration in the rabbit caecum

The neutrophil infiltration into inflamed tissues was monitored by analysis of myeloperoxidase (MPO) activity (Bradley *et al.* 1982). MPO activity in the caecum of DSS-treated rabbits transiently increased at days 5–7 before returning to baseline levels at day 14 (Figure 8a), but due to the low number of animals the change did not reach statistical significance. Analysis of MPO in the ileum and the colon showed no significant differences between colitis animals and the control group (Figure 8b,c). In the ileum, the basal MPO activity in untreated rabbits was higher than in the caecum, but no changes occurred upon exposure to DSS. Overall, our results suggest that the DSS-induced infiltration of neutrophils predominantly localizes in the caecum.

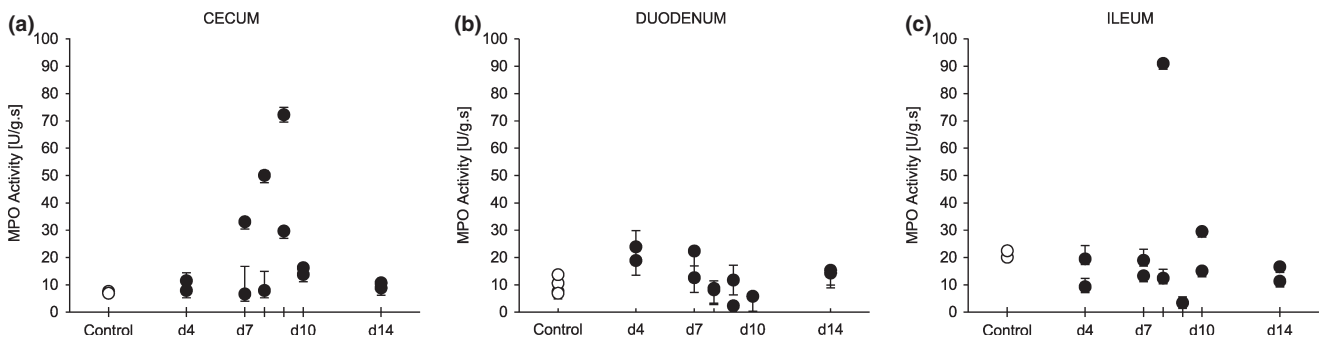

Figure 7 Quantitative RT-PCR showing expression of COX-2 (a, d), IL-6 (b, e) and MMP-1 (c, f) in intestinal epithelial cells (IEC, upper panel) and lamina propria mononuclear cells (LPMC, lower panel) of DSS and control rabbits at day 10 postcolitis induction. Expression is shown relative to GAPDH in the distal colon, $n = 4–9$. Values are given as mean ± SD and difference between groups was tested by two-tailed Student's *t*-test.

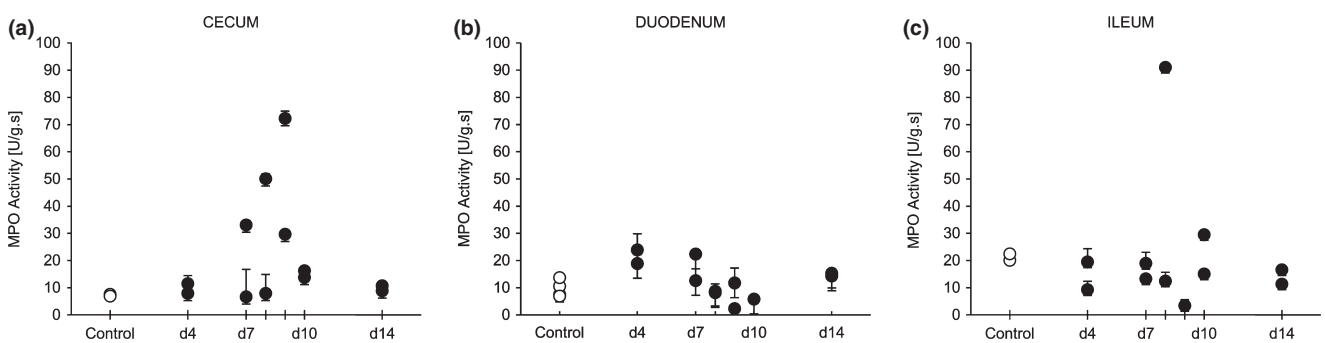

Figure 8 Myeloperoxidase (MPO) was determined as a marker for neutrophil infiltration in the gastrointestinal epithelium. Values for MPO activity in caecum (a), duodenum (b) and ileum (c) samples were normalized for the total protein concentration as determined by BCA assay and for the incubation time (values are represented in arbitrary units U/g s). Dots represent single animals.

Discussion

The helminth parasite *T. suis* has shown promising results for the treatment of IBD in human studies. Unfortunately, efficacy and safety (in particular in immune-compromised subjects) as well as the underlying mechanisms cannot be studied in the well-established mouse and rat models of IBD as the parasite's ova fail to hatch in the intestine of these rodents. TSO are known to hatch in pigs (the natural host), humans and rabbits. As the life cycle of *T. suis* in humans and in rabbits appears similar, a rabbit model of colitis would represent an adequate model for investigations into TSO therapy. The aim of the present study was to develop an IBD model in rabbits by administration of DSS into the daily fluid intake. This study shows that administration of 0.1% DSS for 5 days is sufficient to induce a clear acute inflammation that is localized in the caecum. Localization of the pathology in the caecum makes the DSS model particularly suitable to study the effects of TSO treatment as the caecum is the site of *T. suis* colonization in rabbits.

In accordance with the disease manifestation in other species, the clinical symptoms observed in rabbits were reduced weight gain, reduced food and beverage intake, loose stools and unclean fur (Wirtz *et al.* 2007). The strong reduction in fluid intake began after 5 days only; hence, the daily intake of DSS remained constant throughout the whole induction phase.

The reduction in food intake reflects the response to abdominal discomfort and the disturbances in feeding behaviour that are seen in patients with inflammation of the gastrointestinal tract (Rigaud *et al.* 1994) and are also commonly observed in mouse and rat models of gastrointestinal inflammation (McHugh *et al.* 1993; McDermott *et al.* 2006). To facilitate the evaluation of the disease outcome, we developed a DAI based on the monitoring of the different clinical parameters. Starting from day 4 after DSS administration, rabbits manifested clear symptoms of pathology that gradually worsened. A peak of disease activity was reached at day 9. Afterwards, the DAI decreased and stabilized until the last analysed time point at day 14.

Macroscopical analysis of the internal organs following euthanasia showed no abnormalities. In contrast, histopathological analysis of the intestinal tract revealed that DSS causes an inflammation predominantly localized in the caecum. The other sections of the large intestine and the small intestine remained unaffected.

The caecum localization of the DSS-induced inflammation is also observed in guinea pigs and in the Mongolian gerbil model (Iwanaga *et al.* 1994; Bleich *et al.* 2010). These species possess a functional caecum that is particularly enlarged and provides a niche for the microbial fermentation of cellulose (Snipes 1982, 1997). The caecum localization of the DSS-induced inflammation might be due to an increased permeability of the intestinal barrier to DSS in this particular section of the intestine (Hoshi *et al.* 1996). The localization of the lesions in gerbils has been linked to the increased absorption of sulphated polysaccharides in this particular section of the gerbil intestine, and absorption of DSS in the caecum has also been reported in rabbits (Sharratt *et al.* 1971) and might explain our observations. In accordance with DSS models in other species, DSS treatment induced both a disruption of the mucosal morphology and an infiltration of immune cells (Melgar *et al.* 2005). In particular, the histopathology of the caecum displays crypt loss, epithelial damage and infiltration of immune cells. These manifestations reproduce characteristic traits commonly observed in ulcerative colitis (Okayasu *et al.* 1990). Despite the progressive amelioration of the clinical symptoms after their peak at day 9, the histological damage persists longer and displays some characteristics of chronic intestinal inflammation such as the atypical branching of the crypts.

The initial pathology (day 4–9) presents classical features of an acute inflammation. From day 7 to day 10, we observed a transient increase in neutrophil infiltration into the caecal mucosa accompanied by an increased expression of the pro-inflammatory cytokine IL-12 p35. This transient inflammatory activity correlates well with the peak of the DAI and with the histological findings and suggests an initial T helper 1-driven acute response. The increased neutrophil activity in the caecal mucosa is a common feature with the guinea pig colitis model. However, the described model in guinea pigs was performed with high concentration of DSS (3%) and had a fulminant outcome, with 96% of the animals dying within 96 h (Iwanaga *et al.* 1994).

Genomewide mRNA expression profiling in caecum LPMC and ECs at day 10 showed an enrichment of genes involved in chemotaxis and immune response. In particular, the immune response was characterized by genes involved in Th17 signalling, particularly in epithelial cells. An activation of the innate immune response is a feature shared by both patients with CD and UC. In contrast, Th17-associated cytokines are usually observed in the inflamed mucosa of patients with CD, only. Our analysis further showed enrichment for IL-4-related cytokines that would rather suggest a Th2-type response. This type of response correlates well to the atypical Th2 response (mediated by natural killer cells producing IL-13) observed in patients with UC (Fuss *et al.* 2004). A switch into a Th2-type response has been observed as the colitis matures from an acute towards a chronic phase (Alex *et al.* 2009). The features that appear at later stages of the rabbit colitis might indicate that after an acute phase characterized by severe clinical symptoms, mucosal damage and acute inflammation, the pathology acquires a certain degree of chronicity with a shift towards a Th12 immune response. A long-term analysis is necessary to investigate these preliminary observations and to clarify whether the disease resolves after the acute phase or whether it progresses to chronicity.

In summary, we report the development and characterization of a novel DSS-induced colitis model in rabbits. The initial pathology has an acute nature and is characterized by specific clinical symptoms, histopathological changes and higher mRNA expression of inflammatory markers. Our

model provides a safe and reliable induction of colitis in rabbits that is particularly suitable to study the effects and mechanisms of TSO treatment in IBD.

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IL performed the animal experiments, collected and analysed the samples, performed the genetic analysis and drafted the manuscript. FN performed the animal experiments and collected the samples. KA was involved in sample preparation. AC scored the histology specimens. BT and RG contributed to the conception of the study. GR contributed to the interpretation of data; study concept and design; critical revision of the manuscript for important intellectual content; study supervision. IFW contributed to the interpretation of data; statistical analysis; study concept and design; writing and revision of the manuscript; study supervision.

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4 SECOND MANUSCRIPT

Administration of *T. suis* Ova Protects Immunocompetent Rabbit from DSS Colitis, but is Detrimental to Immunosuppressed Individuals

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Own contribution to the publication:

Animal care, treatment, recording of weight curves (IL)

TSO treatment (IL, FN)

Euthanasia of rabbits, samples collection (IL, FN)

Biochemical assays (IL)

Histological scoring (IL, AC)

Isolation of murine LPMC, IECs (IL)

Quantitative rtPCR (IL)

Analysis of transcriptome data (IL)

Study design (IL, FN, IFW, GR,)

Manuscript writing (IL, IFW, GR)

Figure design and arrangement (IL)

Abbreviations:

CD: Crohn's disease,
DSS: dextran sodium sulphate,
IBD: inflammatory bowel diseases,
IEC: intestinal epithelial cells,
LPMC: lamina propria mononuclear cells,
TSO: *T: suis* ova,
UC: ulcerative colits.

4.1 Summary

BACKGROUND & AIMS: Immunomodulation by helminths can be used to prevent and treat immune related diseases. Clinical trials in IBD with *T. suis* ova (TSO) have shown promising results but the underlying mechanisms and the safety in patients with immunosuppressive therapy have not been investigated so far. We used a rabbit model of colitis to study the efficacy, mechanisms and safety of TSO therapy in immunocompetent and immunosuppressed animals.

METHODS: NZW rabbits received 3x2500 TSO (d1, 14, 21, i.g). Colitis was induced at d26 with 0.1% DSS for 5 days. Symptoms were monitored daily and rabbits were sacrificed at d35. Lamina propria mononuclear cells (LPMC) and epithelial cells (IEC) were isolated from caecal biopsies, for RNA extraction and genome wide expression analysis. For immunosuppression, treatment with cyclosporine (100 ul/kg/day p.o) and methylprednisolone (1 mg/kg/day p.o) was started 14d prior to the first TSO treatment and continued until d35.

RESULTS: TSO treatment significantly reduced the weight loss during colitis (% d10/d0, TSO: +4.23±0.98 vs. vehicle: 0.25±1.22; n=8 per group) and reduced the disease activity index (DAI) at d35 from 1.6±0.8 (vehicle) to 0.7±0.2 (TSO). TSO partially protected the caecal mucosa from the DSS induced morphological changes and reduced the extent of lymphocyte infiltration. Expression profiling showed that the effect of TSO mainly influenced LPMC by dampening innate inflammatory and Th17 responses and by down regulating cell-adhesion pathways.

In immunosuppressed rabbits, TSO treatment exacerbated the DSS induced colitis: The weight loss increased from 0.15±1.20 (vehicle, n=8) to 3.10±1.60 (TSO, n=9). Further, histological examination revealed the presence of larvae in the caecal mucosa and a worsening of the histology score by 50%.
CONCLUSIONS: We show that TSO ameliorates the development of colitis in immunocompetent rabbits, where it modulates LPMC immune responses. This preventive effect, however, is lost in immunocompromised animals, where TSO treatment exacerbates the colitis. Caution should be exercised with regard to TSO treatment in immunosuppressed patients.

4.2 Introduction

The etiology of IBD is complex and is not fully understood. Nonetheless, the increase in IBD incidence in developing nations suggests that the environment plays a critical role in the development of both UC and CD [1]. In particular, the hygiene hypothesis suggests that the increasing hygienic standards of the post-industrial era led to an increase of the incidence of several immune related disorders [2]. Among various factors, a clear inverse correlation exists between the distribution of IBD and that of soil-transmitted helminthic infections [3].

According to the old-friends hypothesis, helminths are the intestine-symbionts that co-evolved with the adaptive immune system and are thereby essential for its proper maturation and functioning [4]. Helminths act on the intestinal homeostasis on several levels: they induce regulatory networks that promote anti-inflammatory immune responses, they modulate the composition of the intestinal microbiota [5] and they increase the permeability of the intestinal epithelium [6].

The immune- modulation exerted by helminths has been proposed for the prevention and treatment of immune-related diseases [7]. The swine parasite *Trichuris suis* is the main helminth species tested in IBD. The trials in human IBD patients have been performed by administration of different doses and dosages of embryonated *T. suis* ova (TSO)[279]. The current view is that *T. suis* can to colonize the human intestine where the larvae mature without reaching sexual maturity [9].

The evidence in support of the efficacy and safety of TSO for the treatment of IBD remains inconclusive [10]. The initial open-label studies showed promising clinical efficacy and safety [11]. Yet, larger multicenter studies failed to show a significant effect of TSO in comparison to placebo in mild to moderate IBD patients [10]. Furthermore, the safety of helminth therapy in immunosuppressed individuals that constitute the majority of the IBD patients is a major concern. Summers and colleagues did not report any severe consequence among the immunosuppressed patients involved in their clinical trials [12]. On the contrary, Kradin *et al.* reported the case of an invasive iatrogenic infection in an immunosuppressed patient [13]. It has to be noted, that the nature of IBD itself might mask the presence of TSO induced symptoms, thereby complicating the assessment of the side effects of TSO-treatment.

To date, the lack of a suitable animal model has hampered detailed investigation into the mechanisms and safety of TSO treatment. We recently developed a colitis model in rabbits by administering 0.1% DSS for 5 days in the drinking water [14] since the course of a TSO infection in the rabbit intestine parallels the one in humans. Here, we use this model to test the efficacy and safety of a preventive TSO therapy in immunocompetent and immunosuppressed animals.

4.3 Materials and Methods

4.3.1 Rabbits

All animal experiments were carried out according to Swiss animal welfare laws and approved by the veterinary office of Zurich (license No. 128-2010 and 231-3013). New Zealand white rabbits (Charles River, Kisslegg, Germany) weighing 1.9-2.1 kg (8-10 week of age) were used for the experiments. Rabbits were maintained single-housed with water and food (standard rabbit maintenance diet – Provimi Kliba AG, Kaiseraugst, Switzerland, hay and straw) *ad libitum* on a 12:12 hour light/dark cycle. Upon arrival, animals were kept for at least 4 days under routine husbandry. Afterwards, drinking water was substituted by organic fennel tea (Hipp, Pfaffenhofen, Germany) *ad libitum*.

4.3.2 *Trichuris suis* Ova Administration

Rabbits were randomly assigned to treatment groups receiving either suspensions of 2500 TSO (embryonated *T. suis* eggs, supplied as ready-to-use inoculation doses) or the vehicle (Phosphoric acid buffer pH 5.0 with 0.05% potassium sorbate) in 3 oral doses at day 1, day 14 and day 21. Animals were fasted overnight prior to TSO administration and sedated with 0.4 ml Hypnorm (VetaPharma, Leeds, UK), *s.c*. The TSO suspension (volume 15 ml) was completely filled in a syringe and administered to the animal using a gastric tube (i.e. Ruesch Katheter, CH14, REF 402101, Kernen, Germany). The animals were fed shortly after administration.

4.3.3 Colitis Induction and Clinical Evaluation

DSS Colitis was induced at day 26 as described previously [14]. Rabbit received DSS (MP Biomedicals, Illkirch, France) dissolved in cold fennel tea at 0.1% w/v for 5 days. Control rabbits received cold fennel tea only. Of every animal weight, food and beverage intake, stool appearance and behavior were monitored daily. A disease activity index was calculated according to Table C.1.

4.3.4 Immunosuppression

Immunosuppressive treatment was started 2 weeks prior to the first TSO gavage. The rabbits received oral administration of cyclosporine (100 µl/kg/day, Sandimmun Neoral Trink Lös 100 mg/ml; Novartis Pharma Schweiz AG) and methylprednisolone (1 mg/kg/day, 6α-Methylprednisolone 21-hemisuccinate sodium salt -lyophilized powder, Sigma-Aldrich, Munich, Germany) for 2 weeks daily, afterwards the dose was reduced to half and then continued until the end of the experiment. Control rabbits received vehicle (12% V/V EtOH). 5 ml of blood were collected from the marginal ear vein prior to immunosuppressive treatment and at day 1, 14, 21 and 35 and sent to the clinic of hematology (USZ, Zurich, Switzerland) for a complete blood count.

4.3.5 Euthanasia and Organ Sampling

Euthanasia was performed at day 35 (if not otherwise described) after sedation with barbiturates with an overdose of ketamine hydrochloride (Vétoquinol, Bern, Switzerland) and xylazine (Bayer, Lyssach, Switzerland). The abdominal cavity was exposed by a midline laparotomy and samples were collected from the ileum, jejunum, duodenum, cecum and colon. For RNA extraction and myeloperoxidase activity analysis, the excised samples (0.5 cm in length) were opened by a longitudinal incision and rinsed with cold PBS. 1 cm^2 sections of the cecum were extensively washed with cold PBS until complete removal of the luminal content. The samples were immediately snap-frozen in liquid nitrogen and stored at -80° C until analysis. For histology, samples (three 0.5 cm^2 sections from different regions of the cecum or one 0.5 cm length section of the other tissues) were collected. The samples were carefully washed and fixed with phosphate buffered 10% formalin solution. For genome RNA isolation caecal samples (2 cm^2) were extensively washed with cold PBS and stored on ice in 5% BSA in PBS until further processing.

4.3.6 Isolation of Caecal LPMC and IEC

The dissected specimens were washed with Ca^+- and Mg^+-free PBS, the caecal fold was removed and discarded. The tissue was cut and incubated in medium containing 20 mM EDTA (Sigma-Aldrich) for 30 min at 37° C on a shaking platform (150 rpm). IECs were detached by vortexing and passing through a 70 µm cell strainer (BD Biosciences, Erembodegem, Belgium). The IEC were washed twice, pelleted, resuspended in RLT buffer (Qiagen, Hilden, Germany), snap-frozen in liquid nitrogen and stored at -80° C for later analysis. The remaining tissue containing LP with muscle layer was collected and incubated in 1 µg/ml collagenase type I CLS (Worthington Biochemical Corp., Freehold, New Jersey, USA) at 37° C on a shaking platform (300 rpm). After 15 minutes incubation, the suspension was vortexed and filtered through a 70 µl strainer. Cells were resuspended in 5% BSA in PBS. The undigested tissue was incubated with fresh collagenase solution for additional 15 minutes. The digestion was repeated three times and the washed LPMC were pooled resuspended in DMEM with 5% FCS. LPMC were purified using Ficoll-Paque PLUS (GE Healthcare Europe GmbH, Freiburg Germany) gradient centrifugation for 40 min at 1200 rpm. The viability of the cells was confirmed by tryptan blue staining. Cells were resuspended in RLT buffer (Qiagen, Hilden, Germany), snap-frozen in liquid nitrogen and stored at -80° C for later analysis.

4.3.7 RNA Isolation and Genome Wide mRNA Expression Analysis

Total RNA was isolated with the Qiacube system using the RNeasy Mini Kit with DNase digestion (Qiagen, Hilden, Germany) to eliminate genomic DNA. RNA integrity and quantity was determined on the Agilent 2100 Bioanalyzer (Agilent; Palo Alto, CA, USA). Samples with an integrity score ≥

6.8 were sent to the Functional Genomic Centre Zurich (FGCZ) for sequencing on the Illumina platform. The fold change (FC) was used to express the changes in average gene expression between studied groups. FC was normalized against the control group. The ENSEMBLE IDs were annotated using BetterBunny augmented annotation and analysis of rabbit genes (http://cptweb.cpt.wayne.edu) [15]. MetaCore™ (Thomson Reuters, http://portal.genego.com) was used to perform network analyses. The following cut-offs were applied to select differentially expressed genes for further analysis: pValue ≤ 0.05 and fold change ≥ |1.0|. Process networks (PN, groups of genes involved in main signaling and metabolic processes in the cell in the MetaCore™ database) were considered significant with a pValue ≤ 0.05 and were further selected on the basis of their relevance to inflammatory bowel diseases pathology.

4.3.8 Histopathological Evaluation of Colitis

After careful dissection and fixation, tissues were embedded in paraffin. Serial sections of 5 µm were cut using a microtome (Carl Zeiss AG, Feldbach, Switzerland) and stained with hematoxylin-eosin (HE). The histological changes in the cecum were quantified in a blinded manner by two investigators (range 1 - 24) for morphological features and infiltration of immune cells (Table C.2).

4.3.9 RNA Extraction and Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, California, U.S.A). Gene expression was determined with a *Taq*Man® Gene Expression Assay (#Oc04097051_m1 IL-6; #Oc04250656_m1 MMP1; #Oc03398293_m1 PTGS2 (COX2); #Oc03398448_m1 SLC15A2; #Oc03397715_m1 TNFα; Oc03823548_s1 ALOX-15A2; Oc03397217_m1 ARG-1; Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was measured as endogenous control (#Oc03823402_g1, Applied Biosystems) and used for calculation of relative mRNA expression by the ΔΔCt method. All samples were analyzed in triplicate.

4.3.10 Faecal and Luminal Microbiota Analysis

Fresh fecal samples were collected ad different time points during the experiments and luminal contents were collected from caecum after euthanasia. Samples were snap-frozen in liquid nitrogen and stored at -80° C for later analysis. DNA isolation was performed using the PowerLyzer PowerSoil Kit (MO BIO Laboratories, Carlsbad, CA USA) using 0.25 g feces or luminal content according to the manufacturer protocol. Approximate yield (ng/ml) was first determined by spectrophotometry using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Samples with DNA concentration > 10 ng/ml were sent to a commercial laboratory for metagenomics bacterial analysis based on 16S rDNA sequencing (Microsynth Balgach, Switzerland).

4.3.1 *Trichuris suis* Detection by PCR

Tissue samples (colon, ileum, caecum, brain, kidney, spleen) were collected after euthanasia and immediately snap-frozen in N_2. Samples were sent to IBR Inc (Matzingen, Switzerland) for detection by PCR. Primers for the ITS2 gene were used (Table 4.1). The ITS2 copy numbers is proportional to the number of *T. suis* larvae.

Table 4.1: Primers and standard used for the detection of *T. suis*.

	Sequence
Forward primer ITS2-FW1	5' - CTGCGGAGAGCGGCTAACT – 3'
Reverse primer ITS2-RW1	5' - ATGTAGCGACGACGTAGCCAACT – 3'
Internal Standard Probe IntStd-TS-P	5' - VIC – TGAAAATGCCAAAGTGACAAG – 3'
***Trichuris suis* Probe** ITS-P1	5' - FAM – CAGTACGGAAGCTGCC – 3'

4.3.2 Statistical Analysis

The data obtained from this study were analysed using GraphPad Prism (version 5.04). If not otherwise indicated, Mann-Whitney *U*-test for two independent samples was used for the comparison of the treatment groups, data are shown as mean ± SD.

4.4 Results

4.4.1 TSO Prevent Colitis in Immunocompromised Rabbits

We first tested whether a pre-treatment with TSO could prevent or reduce the development of DSS induced colitis in immunocompetent rabbits. TSO treated animals were protected from the DSS induced weight loss observed in vehicle (Veh) treated animals (Figure 4.1A).

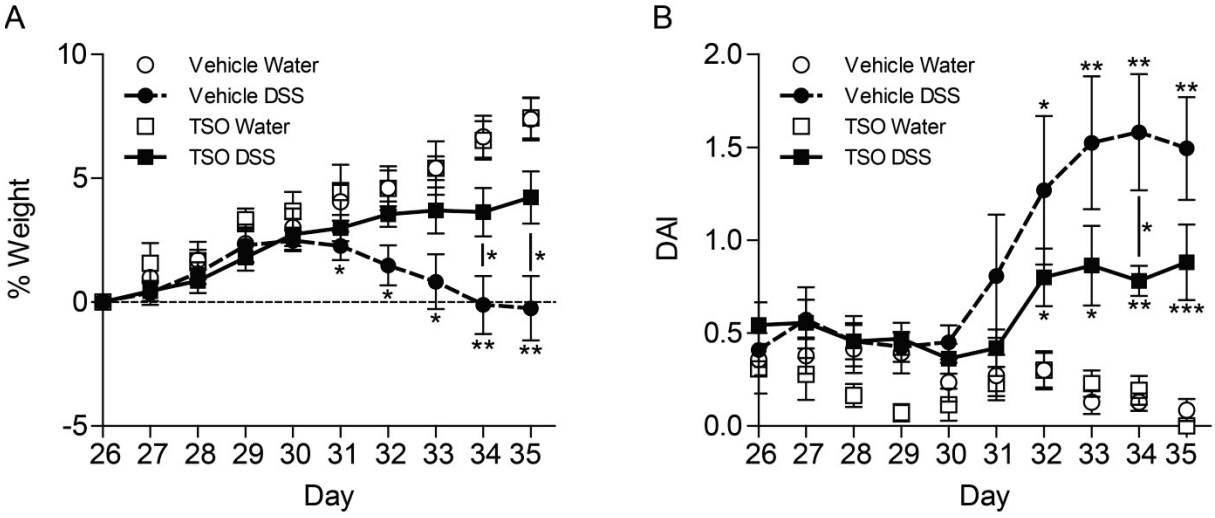


Figure 4.1: TSO treatment ameliorates the outcome of a subsequent induced DSS colitis. NZW rabbits received 2500 TSO at day 1, 14 and 21 (intragastrically). At day 26 colitis was induced by administration of 0.1% DSS in the daily beverage during 5 days. A) Body weight is shown as percentage of individual weight at the time of DSS colitis induction (day 26). B) Clinical symptoms (food and beverage intake, fur cleanliness, weight loss and stool consistency), were monitored daily and summed to a disease activity index (DAI).Bars show mean ± SEM, *P<0.05, **P<0.01, ***P<0.001, two-sided pValue, Mann-Whitney test relative to vehicle water group if not otherwise specified. Data are pooled from 2 independent experiments with 3–4 rabbits in each group in each experiment

Concurrent to weight loss, Veh animals manifested various symptoms including reduced food and beverage intake, unclean fur and diarrhea and at day 35 they had reached an average disease activity index (DAI, Figure 4.1B,) of 1.6±0.8. In contrast, TSO treatment also delayed the onset of the symptoms of 2 days and significantly reduced the disease activity (DAI) at day 34 to 0.7 ±0.2. Importantly, TSO treatment did not induce weight loss and no symptoms where observed in healthy animals (Figure 1B and Figure C.1). When treated with DSS, the control rabbit showed substantial weight loss by day 31 and lost of -0.25% at day 35 (Figure 4.1A).

4.4.2 TSO Prevent Severe Histopathology in the Caecal Mucosa

Histologically, the cecum of TSO treated healthy rabbits did not show any sign of epithelial damage or villous stunting although a mild crypt disproportionation was visible (Figure 4.2A).

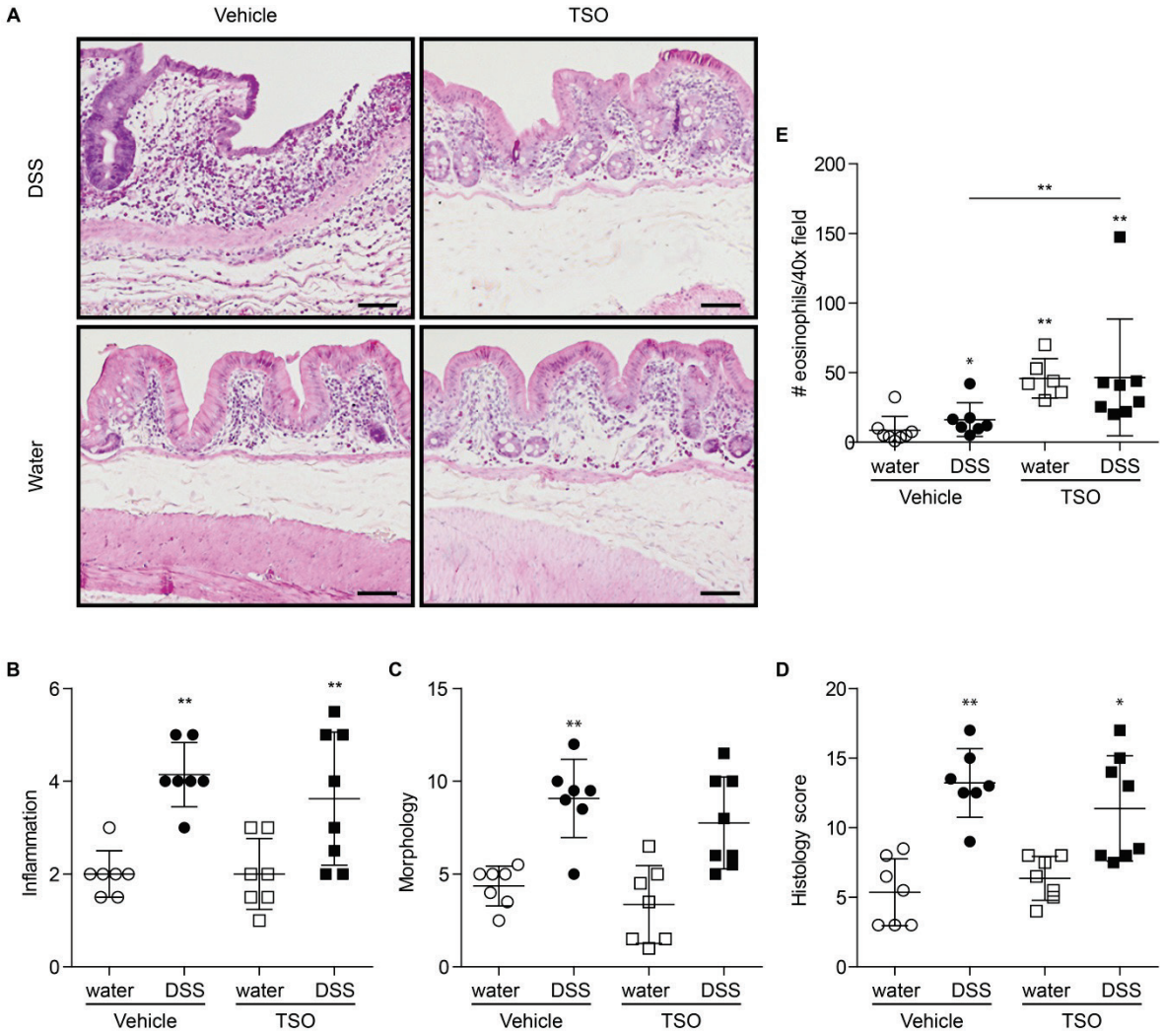


Figure 4.2: TSO treatment reduces the cecum histopathology of the subsequently induced colitis. Caecal samples were collected after euthanasia at day 35. A. HE stained cecum section from vehicle or TSO treated rabbit with or without subsequent colitis induction. Bars correspond to 100 µm Semi quantitative scores of the immune cells infiltrate (B) and morphological changes (C) in the cecum section were summed to a global histopathology score (D). (E) Quantification of the average number of eosinophils within 40x field of cecum sections. Data are pooled from two independent experiments with n≥3 per group. For the scoring, 3 caecal samples per rabbit were collected and evaluated separately in at least three rabbits per group. *P<0.1, **P<0.05, two-sided pValue, Mann-Whitney test relative to vehicle water control if not otherwise specified. Dots represent single animals, bars represent mean ± SD.

In Veh animals, DSS triggered a cecum localized pathology characterized by profound morphological changes and a marked infiltration of lymphocytes into the lamina propria and in the epithelium, leading to a total score of 13.2±2.5 (Figure 4.2B). We found that TSO partially protected the caecal mucosa from the DSS induced morphological changes. In addition, it also reduced the extent of lymphocyte infiltration into the mucosa. No larvae were visible in the histology section. Nonetheless,

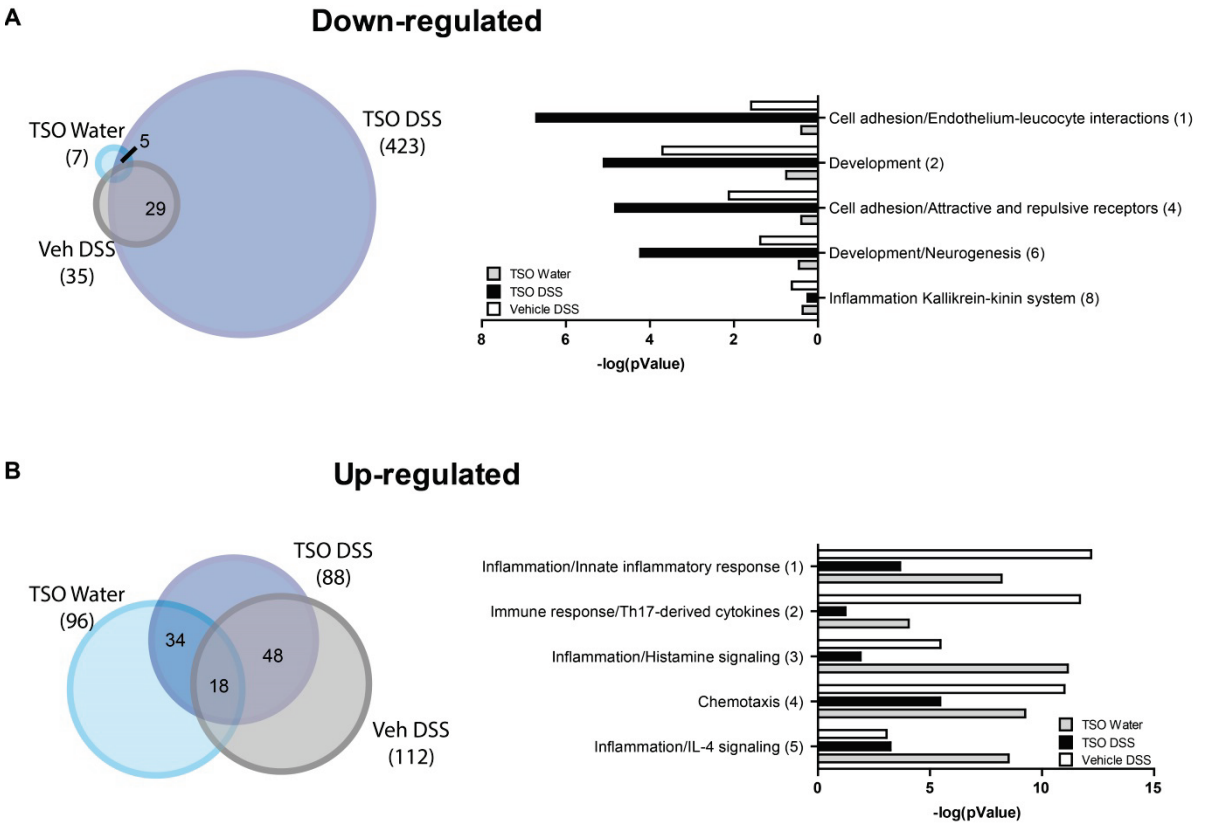
PCR-detection confirmed the presence of *T. suis* in the caeca of 9 out of 12 TSO DSS animals, whereas the TSO water group resulted negative (Table C.3). Blinded semi-quantitative scoring of morphological and inflammatory histological parameters confirmed that TSO treatment reduced the histological score by 14% to 11.4±3.8. As expected, TSO induced a strong infiltration of eosinophils into the lamina propria even in absence of DSS administration (Figure 4.2C).

4.4.3 Transcriptome Analysis of LPMC and IEC

To gain further insights into the mechanism of TSO treatment, we performed gene wide expression analysis on caecal epithelial cells (IEC) and lamina propria mononuclear cells (LPMC) by RNA sequencing (RNAseq). Hierarchical clustering (Figure C.3) revealed distinct expression profiles between EC and LPMC and highlighted significant effects especially in LPMC, whereas the clustering in EC was less clear.

For further analysis, The LPMC genes were filtered to include genes with fold change $|FC|>1$ relative to the level in the vehicle water group with a FDR corrected pValue < 0.05. We identified 511 significantly differentially expressed genes in the TSO DSS animals in comparison to the Veh DSS group. In the TSO DSS animals, 423 genes were over-expressed and 88 were under-expressed.

Genes with significantly altered mRNA levels were analyzed with MetaCore to determine the biological relevance of their differential expression. Enrichment analysis showed that in the healthy mucosa TSO influenced the expression of genes involved in typical features of a parasitic infection such as TCR signaling, phagocytosis, innate inflammatory response and MIF (Macrophage migration inhibitory factor) signaling. Interestingly, 42% of the genes over-expressed by TSO in IEC and LPMC are also over-expressed in the cecum of *T. muris* infected mice [16] (Figure C.4, Table C.4). In the LPMC of DSS treated animals innate inflammatory and Th17 related pathways were significantly over-expressed and that TSO treatment partially prevented this over-expression (Figure 4.3A). Besides, the TSO treatment also led to the down-regulation of genes involved in cell-adhesion and in developmental processes (Figure 4.3B). Differential expression of selected transcripts was verified by rt-qPCR (Figure 4.3C,D).



D

Symbol	FC (pValue) DSS/Ctrl	FC (pValue) DSS TSO/Ctrl
COX-2	1.74 ($8.2 \cdot 10^{-8}$)	<1
IL-6	3.03 ($8.1 \cdot 10^{-8}$)	1.93 ($8.2 \cdot 10^{-6}$)
MMP-1	2.57 ($2.1 \cdot 10^{-3}$)	<1

Figure 4.3: Gene expression signature in caecal LPMC as determined by RNAseq. Hierarchical cluster was used to sort expression profiles for the 4 groups (n=3 per group). Left: Visualization of the down- (A) and up- (B) expressed genes in the TSO DSS, Veh DSS and TSO Water groups in comparison to the control Veh Water group. Right: Top relevant process networks (PN) enriched by genes that were significantly differentially expressed in the LPMC of TSO DSS, Veh DSS and TSO water groups relative to the Veh Water group. (A) Enrichment for the down regulated genes, PN number 3, 5, 7 are not inserted in the graph because not relevant

for the analyzed cell type. (B) Enrichment for the up regulated genes. Numbers refer to the enrichment ranking; genes were selected with fold change FC>|1| and P<0.05. (C) Validation of the genes COX-2, IL-6, MMP-1 in LPMC (data pooled from two separate experiment, with n=3-4 per group) was performed by rt-qPCR, expression changes were quantified with the ΔΔCt method. *P<0.1, two-sided pValue, Mann-Whitney test relative to the vehicle water control, dots represent single animals, bars represent mean ±SEM. The expression patterns reflect those observed by GWAS analysis (D) Genes having a fold change value FC>|1| and P<0.05 were considered differentially expressed genes and were included in the analysis.

To evaluate the impact of TSO on the subsequently induced innate inflammation, innate leukocyte invasion into caecal tissues was assessed by measurement of peroxidase activity. The overall peroxidase activity was increased in the TSO treated animals even in the absence of DSS induced injury (Figure C.2A). Treatment with the eosinophils' peroxidase (EPO) inhibitor aminotriazole (AMT) reduced the peroxidase activity in the TSO treated animals and reveals a trend toward a decreased neutrophil infiltration in the TSO DSS animals although the difference did not reach statistical significance (Figure C.2B).

4.4.4 TSO Exacerbates Colitis in Immunocompromised Animals

Potential aberrant migration of *Trichuris* larvae after TSO treatment of immunosuppressed IBD patients has been a major concern. We therefore investigated the effect of the preventive TSO treatment on DSS colitis in immunosuppressed rabbits. The efficiency of immunosuppressive (IS) treatment by Cyclosporine and Methylprednisolone was confirmed by repeated differential blood counts (Figure C.5C). After 2 weeks of 2 weeks of IS treatment leukocyte count decreased to less than $5 \cdot 10^9$ cells/µl, with lymphocytes being the most affected cell population. Preventive TSO treatment was started, whilst immunosuppression was until the end of the experiment. Interestingly, we observed a reduced weight gain in immunosuppressed animals in comparison to the immunocompetent controls independently of TSO treatment (Figure C.5A). In immunosuppressed individuals without colitis induction, TSO did not induce any clinical symptom and no adverse effects were observed (Figure 4.4A). In contrast, colitis induction was followed by severe weight loss in TSO treated immunosuppressed rabbits. Furthermore, TSO pre-treatment exacerbated clinical symptoms of DSS colitis in immunosuppressed rabbits and led to such a severe course of disease that only six out of nine animals survived until the end of the experiment

Trichuris suis colonizes the caecum of immunocompromised rabbits

Histological examination (Figure 4.5A), showed increased villous stunting and crypt distortion in IS TSO DSS animals as well as enhanced infiltration of lymphocytes into the epithelial layer. As expected, the extent of eosinophil infiltration was enhanced in the two TSO DSS groups (Figure 4.5C). The extent of lymphocyte infiltration into the mucosa (Figure 4.5D) and morphological

distortion (Figure 4.5E) an thus the combined histological score were clearly elevated in comparison to the control group with immunosuppression and colitis induction.

Further examination revealed the presence of larvae in the caecal mucosa of 5 out of 9 IS TSO DSS rabbits. The parasites size and morphological features were characteristic of a late larval or early adult stage, in particular a digestive tube with a tripartite esophagus and well-developed reproductive organs were distinguishable Figure 4.5B). Larvae were not observed in other parts of the intestine (ileum, proximal and distal colon). Still, PCR-detection revealed the presence of *T. suis* in the colon of 4 out of 10 IS TSO DSS and 2 out of 2 IS TSO Water rabbits (Figure 4.4C, Table C.3). The presence of *T. suis* in the colon of IS animals, suggests a failure in the control of the helminth-infection. To test if the parasite could reach sexual maturity in the immunosuppressed host, we regularly tested the feces for the presence of un-embryonated eggs using the fecal sedimentation-flotation method and no positive samples were detected (data not shown). Analysis of immune response markers in the LPMC showed that immunosuppression reduced the increase in the markers of alternative macrophage activation ARG-1 and ALOX-15. Similarly, the increased expression in the SLC15A2 was abrogated, whereas expression of IL-6 increased in the IS TSO DSS animals, although not significantly. On the other hand, the reduction in TNFα observed in the immunocompetent rabbits was still observed (Figure 4.5).

Confirming our previous results the global peroxidase activity but not the MPO specific activity was augmented in the Ctrl TSO DSS group (Figure C.6). On the contrary, analysis of the peroxidase activity in IS animals only showed a small increase after TSO treatment for both the global peroxidase activity and the MPO specific activity. This is accordance with the absence of a clear significant increase in the eosinophilic infiltrate observed with histology.

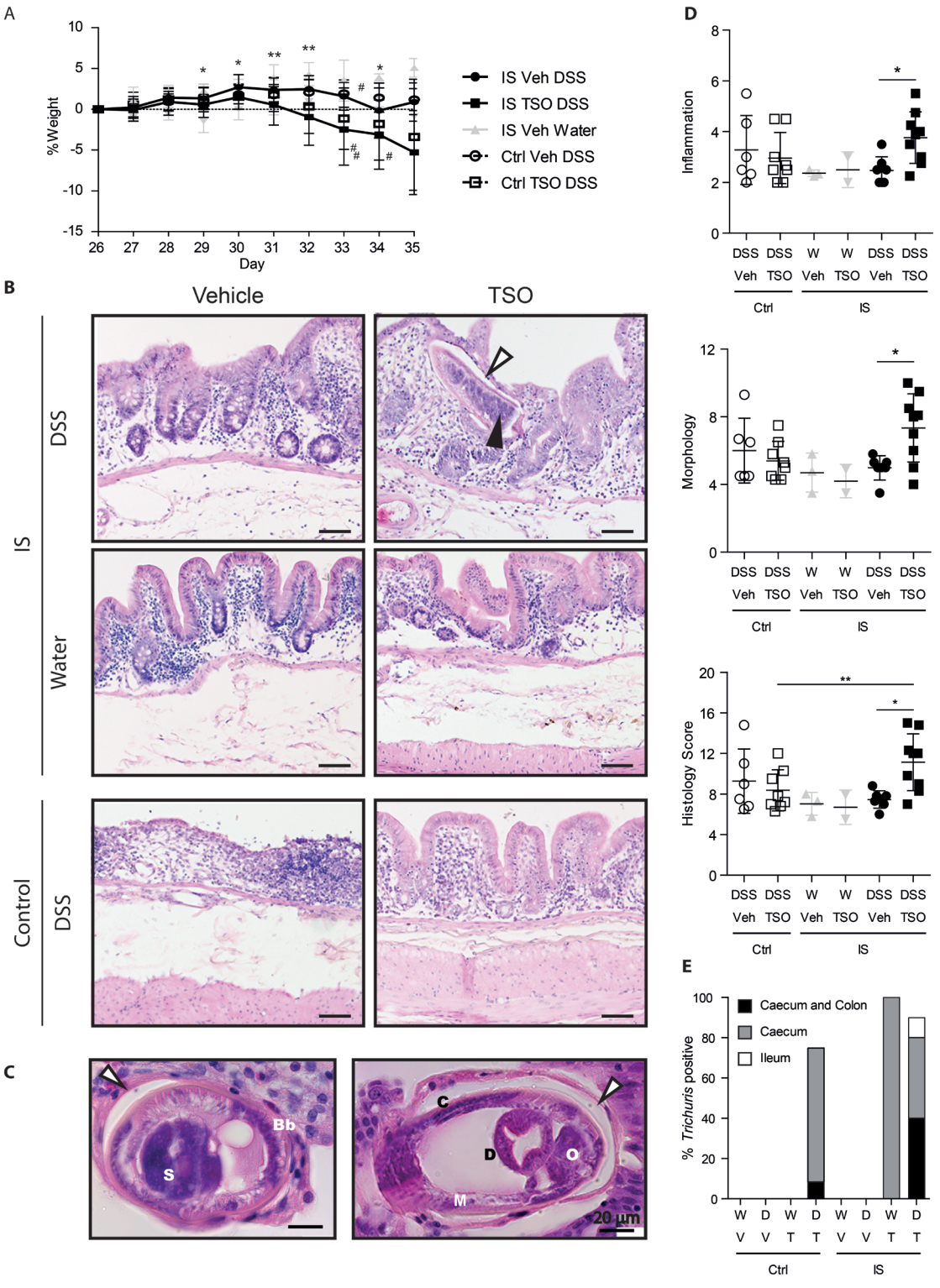


Figure 4.4: Immunosuppression abrogates the protective effects of TSO and exacerbates the DSS induced damage and inflammation of the caecal mucosa. IS was maintained with daily cyclosporine (p.o 100 µl/kg/day) and methylprednisolone (p.o 1 mg/kg/day). 2 weeks after the start of the IS NZW rabbits were treated with 2500 TSO or vehicle at day 1, 14 and 21 (intragastrically). At day 26 colitis was induced by administration of 0.1%

DSS in the daily beverage during 5 days. A. Body weight change was calculated relative to the baseline at the time of DSS colitis induction (day 26). B. representative HE stained specimens of caecal mucosa (day 35). IS: immunosuppressed rabbits, control: immunocompetent rabbits. Bars correspond to 100 µm. 3 specimens per rabbit were assessed. Filled arrowhead: *T. suis* schistosome. C. HE stained section of the caecal mucosa of IS TSO-DSS rabbits showing the presence of adult T. suis. The left panel shows a longitudinal section of the posterior part; the right panel shows a cross section of the posterior end of the parasite. S: stychosome nucleus; Bb: bacillary band; C: cuticle; O: reproductive organs; D: digestive tract; M: muscle layer. Bars correspond to 20 µm. Empty arrowhead: syncytial tunnel derived from the host's caecal epithelium. D: Inflammation score was calculated on the basis of the lymphocytes' infiltrate in the lamina propria and in the epithelium (2-8). Villous stunting, epithelium damage and crypt distortion were scored independently and summed to a morphology score (3-12). Morphology and inflammation score were the summed to a global histology score (5-20). E. Percentage of animals found positive to *Trichuris* in different regions of the gastrointestinal tract (caecum, colon and ileum). RNA was extracted from homogenized tissue specimens. *Trichuris suis* was detected by PCR amplification of the sequence W: water, D: DSS, V: Vehicle, T: TSO, Ctrl: Control, IS: immunosuppressed. (#): number of analyzed animals. Dots represent single animals, bars represent mean ± SD, data were pooled from 3 independent experiments. *P<0.1, **P<0.05, two-sided pValue, Mann-Whitney test between IS Veh DSS (n=8‡) and IS TSO DSS (n=9‡). ‡: n at the start of the experiment. 3 IS TSO DSS and 1 IS Veh DSS animals reached the euthanasia criteria before the end of the experiments and were sacrificed at the indicated time point. # Premature death: 1 IS TSO DSS rabbit was found dead unexpectedly at day 34.

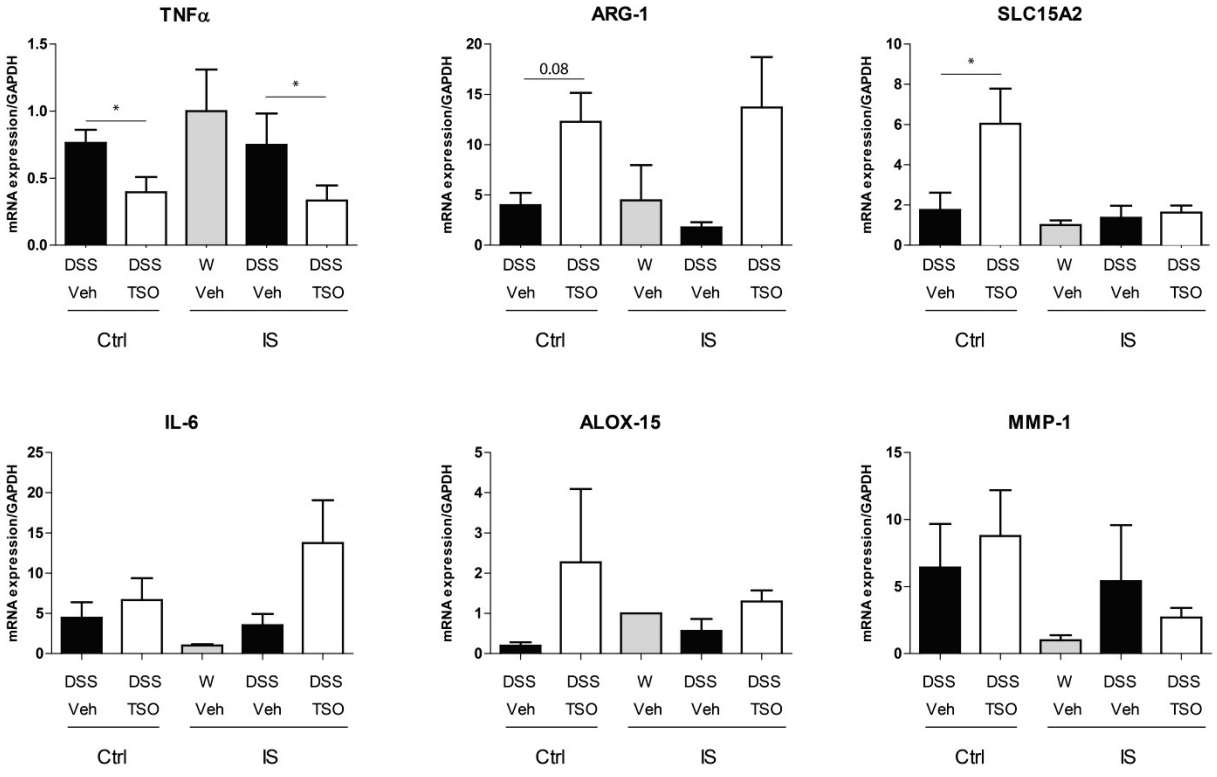


Figure 4.5: Effects of immunosuppression of the expression of different immune response markers in LPMC. LPMC where isolated from the caecal mucosa of control (ctrl) and immunosuppressed (IS) rabbits. Expression of selected genes was determined by qRT-PCR. GAPDH was used as a housekeeping gene. Bars represent Mean±SEM form 2 pooled experiments. *p<0.05, Mann-Whitney test.

4.5 Discussion

The progressive, relapsing, chronic course of inflammatory bowel disease requires a long-term treatment that maximizes the anti-inflammatory action while minimizing the systemic effects. Unfortunately, the performance of the current therapies is low. The first studies on a therapeutic effect of *Trichuris suis* for both UC and CD were promising. Yet, larger multicenter studies could not show a significant effect of TSO in comparison to placebo in mild to moderate IBD patients and the available evidence in support of TSO therapy is currently judged insufficient [10]. Furthermore, the mechanisms and effects underlying TSO therapy at molecular level as well as the safety in immunosuppressed individuals could not be investigated so far due to the absence of a suitable animal IBD model. Here, we studied the efficacy and safety of a preventive infection with *T. suis* ova (TSO) in a recently developed rabbit model of colitis [14]. To date - besides the pig host – *T. suis* has only been shown to hatch in humans, primates and rabbits. We chose to use a rabbit model of colitis[14] since the course of a TSO infection resembles that observed in humans with *T. suis* hatching and colonizing the gastrointestinal tract for 2-3 weeks but without reaching sexual maturity [9].

Consistent with the previous human studies, we found that a preventive treatment with 3 doses of 2500 TSO protected from colitis induced weight loss and reduced the disease activity index and the caecal histology score in our rabbit model of acute DSS colitis. Intriguingly, whole genome transcriptome analysis of caecal LPMC and IEC samples, showed a strong effect of TSO treatment on the lamina propria cells gene expression, whereas the effect on IEC was less marked. This observation, agrees with evidence that *T. suis* excreted or secreted proteins can bypass the EC barrier and directly modulate the lamina propria environment [17]. Recently, Hiemstra et al. showed that *T. suis* produces glycans that reduce the EC permeability thus allowing the passage of soluble compounds to the basolateral side [6]. Among the over expressed genes in caecal IEC, several were involved in the degradation of connective tissues. Beside a role in enhancing the barrier permeability, this might lead to the formation of syncytia that allows the colonization of the cecal mucosa [18]. Several proteins excreted/secreted by *T. suis* have been linked to chemokine, T-cell receptor and TGFβ signaling as well as leukocyte transendothelial migration [6]. Accordingly, we could observe a TSO induced decrease in expression of genes involved in cell-adhesion, endothelium-leukocytes interactions and chemotaxis. Furthermore, TSO treatment also limited the DSS induced increase in expression of genes involved in Th17 and was associated with an increase in expression of genes involved innate inflammation involved in IL-4 and histamine signalling. Whilst these observations largely accord with the typical anti-helminth response, a TSO specific response might occur, especially given the profound differences observed when comparing the *T. suis* transcriptome with those of other well studied helminths [19]. The majority of the IBD patients receive an

immunosuppressive therapy [20]. As our data suggest that TSO treatment predominantly affects infiltrating immune cells, the protective modulation of the immune response could be lost in those patients thus rendering the therapy inefficacious. Furthermore, the safety of a curative TSO infection in immunosuppressed patients is a crucial concern. Although *T. suis* is not a human parasite, an aberrant migration of *Trichuris* particularly in an immunecompromised host with impaired gut barrier function cannot be excluded a priori. In humans, trichuriasis is caused by *T. trichiura* and can be asymptomatic if the worm burden is low. Symptoms usually appear when the host is infested with more than 200 worms and can range from mild digestive tract distress to anemia, dysentery, bleeding, abdominal pain and more generalized effects, such as loss of appetite for food, nausea, vomiting, anemia, peripheral blood eosinophilia, retarded growth and malnutrition [21].

The published clinical trials with IBD patients did not report any TSO induced side effect, even if some of the involved patients were receiving immune-modulatory therapy [8,12,22,23]. Nonetheless, underlying IBD symptoms might mask the consequences of a chronic Trichurias induced colitis[289]. In fact, symptoms were observed in other studies testing a TSO therapy for non-gastrointestinal diseases, even in the absence of an immunosuppressive therapy. In a randomized double-blinded placebo-controlled clinical trial in allergic rhinitis, patients ingesting TSO had a 3 to 19-fold higher rate of gastrointestinal episodes (flatulence, diarrhea and abdominal pain) compared with placebo subjects []25,26]. Similarly, in two small pilot studies in multiple sclerosis, 1 out of 4 [27] and 3 out of 5 [28] patients receiving TSO experienced mild transient symptoms at about 30days after the first dose of TSO.

To date no systematic study of TSO in immunosuppressed individuals has been performed. Consequently, we tested the preventive TSO administration in immunocompromised rabbits. To achieve immunosuppression, we used a combination of cyclosporine and methylprednisolone. Cyclosporine acts rapidly and is effective in the management of severe UC, whereas corticosteroids are used for moderate to severe relapses of both IBD forms [29]. Importantly, we show that the protective effect of TSO is lost in the immunosuppressed individuals where TSO leads to an exacerbation of the pathology. The sudden deaths of IS TSO DSS rabbits highlight the dangers of a helminth therapy in immunosuppressed hosts. Histology revealed the presence of L4 larvae in the cecum of the immunosuppressed rabbits demonstrating a failure in the control of the infection when the intestinal barrier function is damaged. The histological findings resemble those observed in an immunosuppressed CD patient that developed a iatrogenic Trichuris infection after treatment with TSO [13]. Of note, no egg-bearing worms were observed in the cecum of the infected rabbits nor were eggs observed in their faces. This suggests that *T. suis* is not reaching sexual maturity in the cecum of the infected rabbits but rather remains in a pre-adult stage. Our results, provide important

evidence that immunosuppression interferes with the TSO treatment and might predispose towards adverse effects. Based on these initial findings, the interaction between immunosuppression and TSO treatment should be further investigated and caution should be used when treating immunosuppressed IBD patients with TSO or other therapeutic parasites.

4.6 Acknowledgments

Dr Falk Pharma funded this study and participated in the study conception. The Authors take responsibility for the integrity of the data and the accuracy of the analysis. All of the authors were involved in the development and critical revision of the manuscript and decision to submit the manuscript for publication. IL performed the animal experiments, collected and analyzed the samples, performed the genetic analysis and drafted the manuscript. FN performed the animal experiments and collected the samples. AG scored the histology specimens. BT and RG contributed to the conception of the study. GR contributed to the interpretation of data; study concept and design; critical revision of the manuscript for important intellectual content; study supervision. IFW contributed to the interpretation of data; statistical analysis, study concept and design; writing and revision of the manuscript; study supervision. We wish to thank Prof. Felix Grimm for his help with the *T. suis* detection methods.

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5 DISCUSSION

In this work, we describe a rabbit model suitable for the investigation of *T. suis* ova therapy.
Using this model we show that TSO is safe and ameliorate a subsequent colitis in immunocompetent animals. In contrast, TSO exacerbates the colitis and has no protective effect in immunocompromised animals.

5.1 The need of a DSS Model of Colitis in Rabbits

The commonly used mouse and rats models of IBD are not suitable for the study of a therapy with live *T. suis* as the ova fail to hatch in these rodents. Instead, rabbits appear a good translational model since they are transiently colonised by *T. suis* in the caecum as it occurs in humans[227].
Unfortunately, the existing rabbit models appear inadequate for the study of *T. suis* ova (TSO) (Table *5.1*). Acetic acid and TNBS are applied to the rabbit with a rectal enema and induce a pathology localized to the rectum and distal colon. The application of an enema is invasive and requires sedation of the animals. Additionally research using the acetic acid colitis model decline, since the pathology features an initial epithelial cell damage and the immune response is delayed and does not progress to chronicity[290].
We successfully established an acute model of colitis in rabbits by administration of 0.1% DSS in the daily beverage for 5 days. DSS administration induces a caecum localized colitis, whereas no pathology is observed in other parts of the intestine. To permit a longitudinal assessment of the colitis progression we defined a disease activity index and we confirmed that the monitored clinical symptoms correlated well with the extents of the histologic damage.
The pathology included morphological changes such as villous stunting, crypt distortion and villous epithelial injury as well as infiltration of immune cells (particularly neutrophils) into the epithelial layer and the lamina propria. Our DSS model resembles the clinical manifestations and microscopic features of the human pathology (especially UC). Further, the observed changes are similar to those induced by DSS in mice and rats (Table 5.1).
The induction protocol via the daily beverage is simple, has a reasonable time frame and the outcomes appear highly reproducible. Finally, the localisation of the pathology in the rabbit caecum makes the DSS model particularly suitable to study the effects of TSO treatment as the caecum is the site of *T. suis* colonisation in rabbits.
In other species (mouse, rat, mongolian gerbil and guinea pig), 2-5% DSS is the standard dose for induction of acute colitis. The rabbit intestine appears more sensitive as higher doses caused an acute and fulminant colitis. This increased sensitivity is also observed in the acetic acid model that is

induced in rabbits by a 0.5% whereas in rat and mouse it is induced by a 3-5% acetic acid enema (Table 5.1).

Table 5.1: Chemically induced colitis in the rabbit

Model	*Application*	*Sedation*	*Localisation*	*Start-peak*	*Dose (rabbit)*	*Dose (mouse/rat)*	*Histology*
Acetic Acid	Rectal enema	Yes	Colon	1h-1d	0.5% in 1ml	3-5% in <1ml	loss of IEC, loss of GC, crypt abscesses, edema, neutrophil influx
TNBS	Rectal enema	Yes	Colon	1h-7d	40mg in 25% EtOH	20-80mg in 20-50%EtOH	Crypt abscesses, ulceration, crypt distortion
DSS	Oral in daily beverage	No	Caecum	5d-14d	0.1% DSS, 5d	2-10% 5-7d	Crypt loss, epithelial damage, immune cells infiltration

Expression profiling of the caecal tissues at day 10 revealed an enrichment of IBD related genes in both lamina propria mononuclear cells and intestinal epithelial cells. Most genes belonged to pathways involved in chemotaxis and immune response. In particular, genes involved in Th17 signalling and innate immunity were enriched in the IEC transcriptome.

Activation of the innate pathway is common in IBD patients. On the other hand, the IL-23/Th17 axis has pathogenic relevance in CD only[291]. For example, transcription and translation of Th17 related cytokines are typical in active CD lesions and $Th17^{+}$ $CD4^{+}$ lymphocytes are frequent in the lamina propria of CD patients[42].

Interestingly, we also observed an enrichment of genes involved in a Type 2 response. This type of response is rather observed in UC patients, whose lamina propria T cells produce greater amounts of IL-13[46]. Our observation, correlate with the cytokine profile observed in murine DSS colitis: following an initial Th1/Th17 response a Th2 response develops as the colitis progresses toward chronicity[183].

Our results suggest that in rabbit DSS colitis, an initial acute phase characterized by severe clinical symptoms, mucosal damage and acute inflammation precedes a chronic phase with a shift toward a Th2 immune response. A long-term analysis would be necessary to confirm these preliminary observations and to clarify if the disease resolves after the acute phase or if it progresses to chronicity.

An important caveat of DSS colitis in mouse and rat is the dispensability of B and T cells[292]. Although we did not directly address their role in rabbit, the transcriptome analysis suggests a secondary role of adaptive immunity also in our model.

5.2 TSO in Immunocompetent Animals

Using the rabbit model we demonstrate the efficacy and safety of a preventive administration of TSO against DSS colitis. The animals receiving 3 doses of 2500 TSO did not experience gastrointestinal side effects (diarrhoea, bloody faeces).

Importantly, *T. suis* infected rabbits were protected from DSS induced weight loss and developed milder clinical symptoms. Our results confirm the positive outcomes of TSO treatment in IBD patients and the safety of its administration in immunocompentent individuals[287,285].

At the histological level, *T. suis* reduced the extent of morphological damage and lymphocytes infiltration in the lamina propria and in the intestinal epithelium. The semi-quantitative scoring of the histological damage does not reach statistical significance; this might be due to the limited number of animals included in the experiment.

In the TSO-DSS group, half of the rabbits did not experience a clear amelioration of the microscopic features. Non-responders are also present in clinical trials with IBD patients where a double blind clinical study has shown a non-response rate of 44%[282]. The New Zealand White (NZW) rabbits used for this study belong to an outbred stock: a higher variability is not surprising and the subdivision in responder/non-responder groups might be ascribable to inter-individual genetic variation.

As expected, eosinophils were recruited to the caecum of *T. suis* infected animals. Infiltration of eosinophils into infected tissues has been observed in TSO treated patients[125] and in *T. trichiura* treated macaques[278]. Generally, eosinophilia is a common feature of parasitic infections, although its role is not completely understood[293,294].

To explore the mechanisms by which TSO exert their protective effect, we performed a transcriptome analyses on the caecal epithelial cells (EC) end lamina propria mononuclear cells (LPMC) at day 10 post colitis induction by using next generation RNA sequencing (RNAseq). TSO induced distinct profiles on IEC and LPMC and exerted a stronger effect on LPMC then on IEC.

In the healthy mucosa TSO induced changes typically observed upon parasitic infection such as TCR signalling, phagocytosis, innate inflammatory response and MIF signalling. It is noteworthy that 26% of the genes up-regulated by TSO in IEC and LPMC are also up-regulated in the caecum of *T. muris* infected mice[230], confirming the similarities among different Trichuris species[230;231].

In the colitis rabbits, TSO counteracted the DSS induced up-regulation of several genes involved in Th17 pathways and in innate immunity. Further, TSO also affected genes involved in cell-adhesion, chemotaxis and developmental processes.

The stronger influence exerted by *T. suis* on LPMC might be a consequence of several factors:

1. The effect of *T. suis* on the intestinal epithelium occurs in the early phases of the infection when the larvae start colonising the caecum. Our analysis was performed 10 days after the

last TSO administration and might have "missed" the early phases of the IEC-*T. suis* interaction.

2. Some *T. suis* secreted products can bypass the intestinal epithelium and directly penetrate the mucosa where they act on LPMC. This is in agreement with the finding that *T. suis* glycans increase IEC permeability and allow the passage of soluble compounds to the basolateral side.
3. DSS induced damage of the intestinal epithelium hinders the detection of the changes induced by TSO.

A longitudinal observation of the effects induced by *T. suis* on the caecal mucosa would help to reveal the complex cross talk between *T. suis* and the intestinal mucosa during the various phases of the infection.

5.3 TSO in Immunocompromised Animals

The whole genome transcriptome study shows that *T. suis* influence the response of LPMC to the DSS induced injury. In the following studies, we investigated whether the administration of TSO would be as efficacious and safe in an immunocompromised host.

To this end we developed an immunosuppression protocol for NZW rabbits by a combination of cyclosporine and methylprednisolone. Cyclosporine inhibits the NF-AT mediated IL-2 production and thus affects the proliferation of T lymphocytes. Methylprednisolone is a corticosteroid and has a broader effect: it reduces NF-κB activation and down-regulates pro-inflammatory cytokines. The reduced weight gain observed in the immunosuppressed groups is a common side effect observed in cyclosporine treated rabbit[295].

Prior to the administration of DSS, TSO did not cause any side effects in the immunosuppressed rabbits. Our observation, suggest that in presence of an intact intestinal epithelium an immunosuppressed host can control the *T. suis* infection.

In contrast, in presence of a DSS-induced colitis, TSO exacerbated the weight loss and further symptoms and can lead to fulminant colitis and death in immunocompromised animals.

Similar to the report of an iatrogenic infection in a CD patient by Kradin[283], histological examination revealed the presence of late stage larvae in the caecum of the immune-suppressed rabbits supporting a failure in the control of the iatrogenic infection when the intestinal barrier function is damaged.

The lack of beneficial effects and the worsening of colitis might be a consequence of the different developmental stages reached by *T. suis* in the immunocompromised host. The transcriptome of *T. suis* changes as the larvae progress toward the adult stadium[231], and we can hypothesize that the interactions of the early larval stages exerts a beneficial modulation of the intestinal niche. This

positive effect would then be lost as the infection progresses towards chronicity as observed in susceptible strains infected with *T. muris*[230,239] (Figure 5.1).

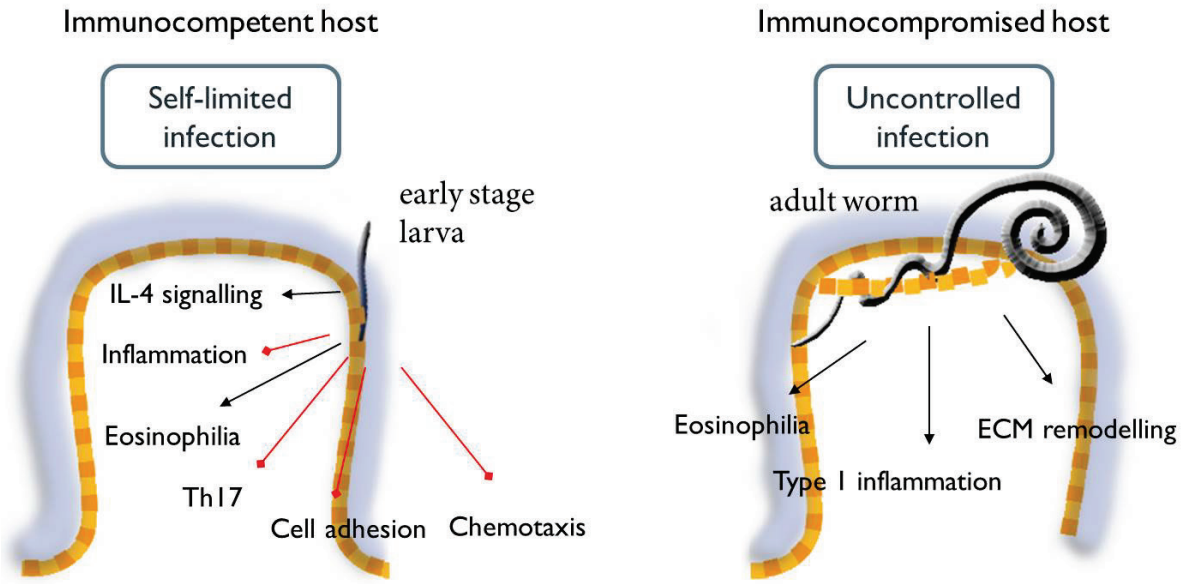

Figure 5.1: Modulation of the caecal niche by different stages of *T. suis*. In immunocompetent hosts infection with *T. suis* is cleared before the parasite reaches a late larval stages. *T. suis* larvae exert a beneficial effect on the inflamed mucosa and down-regulates the expression of several colitogenic pathways. In immunocompromised hosts *T. suis* develops further and the protective effect is lost. A strong remodelling of the caecal mucosa occurs and the persistent Trichuris infection leads to an exacerbation of colitis. Black arrows indicate stimulatory pathways; red diamond-tipped arrows indicate inhibitory pathways.

The existing studies in IBD, reported no side effects, even in immunocompromised patients (Table 1.10). Yet, the limited number of patients and the absence and the nature of these studies call for further larger double blind studies with TSO[286].

To date no study has specifically addressed the concerns of safety and efficacy in the immunocompromised patients.

Our results provide important evidence that immunosuppression interferes with the control of *T. suis* and can predispose towards serious adverse effects.

5.4 Alternative Approaches to the Live Worm Therapy

Our study highlights the promises of helminth treatment for IBD and yet, at the same time, evidences its perils. Helminth therapy concept warrants more investigation before a widespread clinical use can be initiated.

Besides the observed beneficial effects, our results evidence the variability of the individual response to TSO. The interaction between Trichuris and host is intricate and encompasses all the components of the mucosal niche, from bacteria to immune cells.

Our results show, that TSO therapy can lead to a noxious iatrogenic infection, especially when both the host barrier and immune defences are compromised.

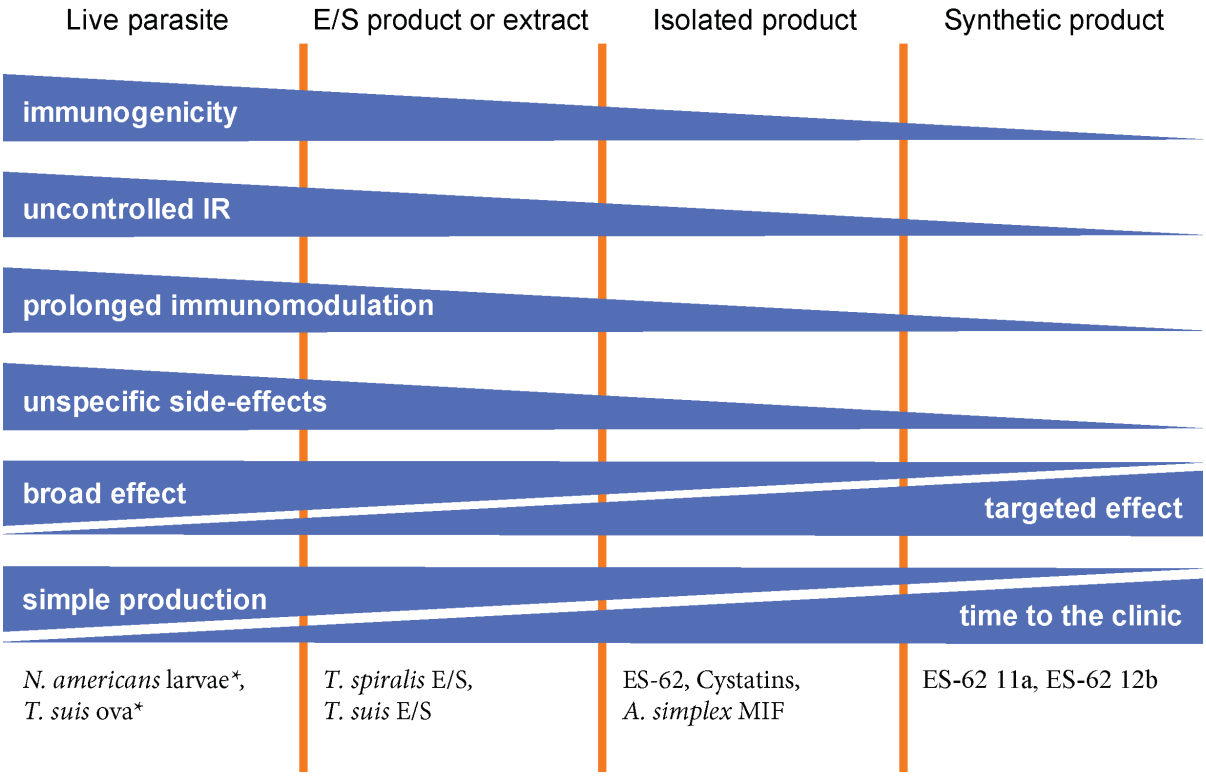

Figure 5.2: Different approaches to helminth therapy. Helminth therapy can be applied by using either live parasites or parasite derived products. Extracts, Lysates or E/S products can be further purified to identify and isolate selected products with improved characteristics. As an alternative, parasite molecules can be used as template to generate synthetic drugs.

As we have remarked in the introduction, a therapy with live parasites is not the sole option, and alternative approaches can limit the drawbacks of a live infection (Figure 5.2). E/S products from *T. suis* and other parasites possess immune-modulatory properties[103;104] and can be used preventively or as a treatment in animal models of several diseases (chapter 1.4).

E/S products are not completely risk free: they include a variety of components that are still poorly characterized and might cause counterproductive effects or possess immunogenic potential. Further, the concentrations of the "beneficial" component in the E/S might be low and thus require the administration of large doses. For example, the immune-modulatory serpins only constitute the 0.15% of the whole *T. suis* secretome[232]

The identification and isolation of active compounds from E/S products might allow a pharmacologically reasonable dosage and facilitate the translation to the clinic. This approach has proven efficacious in different animal models. The glycan LNFPIII from *S. japonicum* possesses beneficial effects in EAE and in a mouse model of psoriasis[118,139]. Another example is the filaria derived glycoprotein ES-62, that is protective in allergy and rheumatoid arthritis models[100,131]. Isolating single components of the E/S products allows for selection and concentration of safe and efficacious molecules but limits the range of the targeted pathways. Further, this method probably requires frequent administration of the immune-modulatory product.

A smart solution to this problem comes from a study in the DSS colitis model where an E/S protein was cloned into probiotic bacteria to target its delivery into the intestine and ensure a prolonged release[219]. An *E. coli Nissle* strain modified to secrete AvCys ameliorates colitis in both a mouse and a pig model proving that this system is not only effective in small rodents but can also be applied in larger animals[219].

Many E/S products with immune-modulatory effects are large, potentially immunogenic molecules and appear unsuitable as a standard drug. To avoid this problem, helminth products can be used as a basis for novel drugs with improved characteristic. The immune-modulatory site of E6-62, a phosphorylcholine, was used as a basis to construct a library of small-molecule analogues that were screened in vitro for immune-modulatory properties. This approach allowed the identification of two drugs called 11a and 12b that possess a preventive effect in CIA and asthma models[101,102]. This approach is surely promising and for sure warrants further trials with other helmitnths' E/S products. Yet, the development of these analogue drugs is laborious and they will not be readily available to the clinic in the near future.

6 CONCLUDING REMARKS AND OUTLOOK

In this work we show that preventive treatment with *Trichuris suis* ova (TSO) exerts a preventive effect on the development of a DSS induced colitis in a rabbit IBD model.

This effect is only observed in immunocompetent animals, and TSO administration is detrimental to immunocompromised animals as it exacerbated disease.

In our opinion, our results possess a high relevance to the clinic, since the majority of IBD patients are immunosuppressed. Our DSS colitis model mimics the acute flares of colitis in IBD patients and the obtained results suggest that TSO administration during the remission period can prevent a relapse of the disease.

We have not yet addressed the effect of TSO in a chronic model. In mice and rats the DSS model can be easily adapted to generate a chronic colitis. It is reasonable to hypothesize that a repeated administration with a slightly reduced DSS dose should similarly allow for induction of chronic colitis in rabbits.

A further study of a therapeutic administration of TSO would be of interest, but is complicated by the delay between the administration of TSO and the colonisation of the caecum.

As an alternative, *T. suis* extracts would easily allow the investigation of a therapeutic administration.

The mechanisms of action of helminths and in particular of *T. suis* are still open to question, but probably involve several pathways and cellular processes.

In this work, we have mainly addressed the mechanisms of TSO action at the transcriptional level. We can thus only speculate that E/S products secreted in the caecum cross the epithelial barrier and modulate the action of LPMC.

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Publications

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APPENDIX A

Summary of the studies on the therapeutic use of helminth infection or helminth derived products in rodent model of inflammatory bowel diseases (Figure A. 1).

Figure A. 1: Summary of the studies on the therapeutic use of helminth infection or helminth derived products in rodent model of inflammatory bowel diseases.

Author	*Year*	*Effect*	*Species*	*Parasite*	*Component*	*Disease Model*	*Timing*
			Ancylostoma (Nematode)				
Cancado	2001	Live worms not required; attenuation of colitis, live worm not required	mouse	*A. ceylanicum*	ES and crude extracts	DSS colitis	Concomitant
Ruyssers	2009	Attenuation of colitis. Dose-dependent decrease of inflammation and MPO activity	mouse	*A. caninum*	ES	TNBS colitis	Therapeutic
			Anisakis (Nematode)				
Cho	2011	Attenuation of colitis. Lower inflammatory CK production and higher Treg frequencies. *A. simplex* MIF II induced in vitro expression of IL-10 by EC, DCs, and fibroblasts and TGFβ by fibroblasts.	mouse	*A. simplex*	larvae	DSS colitis	Preventative
			Acanthocheilonema (Nematode)				
Schnoeller	2008	Attenuation of colitis. (Mac dependent)	mouse	*A. vitae*	cystatin	DSS colitis	Concomitant
Whelan	2014	Attenuation of colitis, ↑ $Foxp3^+$ Tregs, ↓ local IL-6 and IL-17A production, ↓ MIP-1α/β, MCP-1/3, and RANTES, ↓ inflammatory Macs in colon	mouse	*A. vitae*	*E. coli* producing *A.viteae* cystatin	DSS colitis	Concomitant
			Brugia (Nematode)				
Kron	2013	Attenuation of colitis, ↑IL-10 production (by $CD3^+$ and CPG and LPS stimulated splenic cells)	mouse	*B. malayi*	asparaginyl-tRNA synthetase	T cell transfer colitis	Therapeutic
			Hymenolepis (Cestode)				
Wang	2015	Attenuation of colitis. ↑ TGFβ by splenic follicular $CD19^+$ B cells. IL-4 and IL-10 indep, T and B cell indep (*Rag KO* mice). Mac dep.	mouse	*H. diminuta*	$CD19^+$ B cells from infected mice	DNBS-, oxazolone-, DSS-colitis	Concomitant
Reardon	2001	Assumed Th2 response. Normalization of colonic ion transport, but no improvement in colonic histopathology	mouse	*H. diminuta*	larvae	DSS colitis	Preventive Therapeutic

Author	Year	Effect	Species	Parasite	Component	Disease Model	Timing
			Hymenolepis (Cestode)				
Hunter	2005	Preventive: Augmented Th2 response (↑ *Il-4*, ↑ *Il-10* mRNA expression), attenuation of colitis. Therapeutic: IL-10 required for protective effect. Enhanced recovery of colitis	mouse	*H. diminuta*	larvae	DNBS colitis	Preventive
Hunter	2005	No effect on colitis. Not specified.	rat	*H. diminuta*	larvae	DNBS colitis	Preventive
Hunter	2007	Worsening of colitis. Augmented Th2 response (↑ IL-4, ↑ IL-5, ↑ IL-13, ↑IL-10), changes in Treg response.	mouse	*H. diminuta*	larvae	Oxazolone colitis	Preventive
Hunter	2010	Attenuation of colitis. ↑Mobilization of alternatively activated macrophages.	mouse	*H. diminuta*	larvae	DNBS colitis	Preventive
Melon	2010	Attenuation of colitis. Diminished Th1 response (↓TNF, ↓IFNγ), augmented Th2 response (↑ IL-4, ↑ IL-10, ↑eosinophils), augmented Treg response (↑ IL-10).	mouse	*H. diminuta*	larvae	DNBS colitis	Preventive
			Heligmosomoides (Nematode)				
Elliott	2004	Attenuation of colitis. Diminished Th1 response (↓ IL-12, ↓IFNγ), Th2 induction. (↑IL-13), Treg response (↑*Foxp3* mRNA expression). Protection can be transferred with T cells from worm-infected donors.	mouse (*Il-10 KO*)	*H. polygyrus*	larvae	*Il-10 KO* spontaneous colitis	Therapeutic
Sutton	2008	Attenuation of colitis. Diminished Th1 response (↓*Tnf*, ↓*Ifnγ* mRNA expression), augmented Th2 response (↑ *Il-4*, ↑ *Il-13* mRNA expression), mast cell-mediated effects.	mouse	*H. polygyrus*	larvae	TNBS colitis	Preventative
Urban	1991	$CD4^+$ but not $CD8^+$ T cells regulate the IgE response and protective immunity in mice.	mouse	*H. polygyrus*	larvae	healthy	na
Fox	2000	Attenuation of colitis. ↓inflammation, gastric immune responses and gastric atrophy. ↓IgG1, IFNγ, TNF, IL-1B, IP-10, MIP-1β and RANTES ↑IgE, TGFb	mouse	*H. polygyrus*	larvae	*H. felis* colitis	Preventive
Chen	2005	↑Th2 response (IL-4, IL-5, IL-10), ↑Treg response (IL-10) Changes in Th1 response (↓IFNγ, ↑TNF), STAT6-mediated mechanism. **worsening of colitis**	mouse	*H. polygyrus*	larvae	*C. rodentium* colitis	Preventive

Author	Year	Effect	Species	Parasite	Component	Disease Model	Timing
			Heligmosomoides (Nematode)				
Chen	2006	$CD11c^+$ DC expansion and ↑ IL-10 mRNA expression. Transfer of parasite-primed DC exacerbates colitis. No exacerbation with *Il-10 KO* DCs.	mouse	*H.polygyrus*	larvae	*C. rodentium* colitis	Preventive
Metwali	2006	Attenuation of colitis. $CD8^+$ Tregs required, act independently of IL-10 or TGFβ signalling	mouse	*H. polygyrus*	larvae	*Il-10 KO* T cell transfer colitis	Therapeutic
Setiawan	2007	Attenuation of colitis. Diminished Th1 response (↓ IL-12p40, ↓IFNγ), augmented Treg response (↑ IL-10) IL-10R blockade in vitro restores IFNγ and IL-12p40 production by mucosal cells.	mouse	*H. polygyrus*	larvae	TNBS colitis	Preventive
Elliott	2008	Attenuation of colitis. Suppression of IL-17 production. IL-4 and IL-10 block IL-17 production by T cells. IL-10 alone is not sufficient.	mouse	*H. polygyrus*	larvae	*Il-10 KO* T cell transfer colitis	Therapeutic
Massacand	2009	*Tslrp* KO normal Th2 cell differentiation, protective immunity and memory responses. ES antigen directly suppressed IL-12p40 production by DC	mouse	*H. polygyrus,*	larvae and ES antigen	healthy	na
Ince	2009	No effect on colitis. IL-10 secretion requires intact T cell TGFβ signaling.	mouse	*H. polygyrus*	larvae	TGFβRII DN colitis	Preventive
Hang	2010	Attenuation of colitis. Phenotypical changes of DC (↓CD80 and CD86, ↑plasmacytoid dendritic cell Ag-1 and CD40). (↓IFNγ, IL-17). Modulation of innate immune cells is sufficient to suppress colitis weeks after abrogation of H. polygyrus infection	mouse	*H. polygyrus*	larvae	*Il-10 KO* T cell transfer colitis	Preventive Therapeutic
Walk	2010	Shift in the abundance and relative distribution of bacterial species in the ileum of mice. ↑ Lactobacillaceae abundance	mouse	*H. polygyrus*	larvae	healthy	na
Wang	2010	Worsening of colitis. Involvement of Th2 response (↑ IL-5 and ↑eosinophils recruitment).	mouse	*H. polygyrus*	larvae	Oxazolone colitis	Preventive
Li	2011	Selectively induce expansion of the $D11c^{low}CD45RB^{mid}$ regulatory DC subset that promotes development of $Foxp3^+$ $IL\text{-}10^+$ Treg cells.	mouse	*H. polygyrus*	larvae	healthy	na

Author	Year	Effect	Species	Parasite	Component	Disease Model	Timing
			Heligmosomoides (Nematode)				
Blum	2012	Attenuation of colitis. Block OVA IFNγ/ IL-17 responses of LPMC isolated from colitic animals through direct contact. Regulatory T cell idep. Block gut Ag-specific IFNγ/ IL-17 T cell response	mouse	*H. polygyrus*	Transfer of DC from infected RAG mice	*Il-10 KO* T cell transfer colitis	Therapeutic
Donskow-Lysoniewska	2012	Attenuation of colitis. ↑Macs infiltration (↑ IL-1β, TNF, IL-6), ↑expression of MOR1, POMC and β-endorphin	mouse	*H.polygyrus*	larvae	DSS colitis	Therapeutic
Leung	2012	Attenuation of colitis. ↓IFNγ, ↓ IL-17, induction of $Foxp3^+$ Treg cells, ↑ IL-10 from non-T cells	mouse	*H.polygyrus*	larvae	Antigen-driven colitis	Preventive
Hang	2013	↑ $Foxp3^+$ T cells (mLN). $Foxp3^+IL\text{-}10^-$ T cell transfer form infected *Il-10 KO* mice : attenuation of colitis and reconstitution of $Foxp3^+IL\text{-}10^-$ and $Foxp3^+IL\text{-}10^+$ T cell subsets	Mouse	*H.polygyrus*	larvae	*Il-10 KO* T cell transfer model	Preventive
			Nippostrongylus (Nematode)				
Massacand	2009	*Tslrp KO* normal Th2 cell differentiation, protective immunity and memory responses. ES antigen directly suppress IL-12p40 production by DC	mouse (*Tslrp KO*)	*N. brasiliensis*	larvae and ES antigens	healthy	na
			Schistosoma (Trematode)				
Heylen	2014	Attenuation of colitis. ↓IL-17A producing T cells; ↑ IL-4 producing T cells	mouse	*S. japonicum*	soluble worm proteins	T cells transfer colitis	Therapeutic
Heylen	2015	Attenuation of colitis (stronger in preventive). ↓*Ifnγ* and *Il-17a* expression; ↑ *Il-4* expression in the colon	mouse	*S. japonicum*	soluble eggs proteins	T cells transfer colitis	Preventive Therapeutic
Zhao	2009	Attenuation of colitis. ↓IFNγ, ↑ IL-10, ↓*Tlr4* mRNA expression related to ↓IFNγ and ↑ IL-10.	mouse	*S. japonicum*	eggs	TNBS colitis	Preventive
Xia	2010	Attenuation of colitis. Diminished Th1 response (↓TNF, ↓IFNγ), maintaining epithelial barrier function trough augmented tight junction proteins	mouse	*S. japonicum*	eggs	TNBS colitis	Preventive
Elliot	2003	Attenuation of colitis. ↓IFNγ, ↑ IL4, ↑ IL-10. STAT6 dep.	mouse	*S. mansoni*	eggs	TNBS colitis	Preventive

Author	Year	Effect	Species	Parasite	Component	Disease Model	Timing
		Schistosoma (Trematode)					
Moreels	2004	Attenuation of colitis. ↑ IL4, changes in smooth muscle contractility	Rat	*S. mansoni*	larvae	TNBS colitis	Concomitant
Smith	2007	Worsening of colitis. Not specified	mouse	*S. mansoni*	eggs	DSS colitis	Preventive
Smith	2007	Attenuation of colitis. Induction of F4/80^{+}CD11b^{+}CD11c^{-} macrophages.	mouse	*S. mansoni*	larvae	DSS colitis	Preventive
Bodammer	2010	Attenuation of colitis. Diminished Th1 response (↓TNF, ↓IL2 mRNA expression), diminished Th2 response (↓ *Il-4* mRNA expression).	mouse	*S. mansoni*	larvae	DSS colitis	Preventive s
		Trichuris (Nematode)					
Massacand	2009	TSLP reduces IL-12p40 production and treatment of *Tslrp KO* animals with neutralizing anti-IL-12p40 mAb reversed susceptibility and ↓IFNγ production. ES has no effect on IL-12p40 production by DC.	mouse (*Tslrp KO*)	*T. muris*	larvae and ES antigens	healthy	na
Taylor	2009	Ab neutralization of TSLP or deletion of the *Tslrp* resulted in defective expression of Th2 CK and persistent infection. Susceptibility: ↑ expression IL-12/23p40, IFNγ, and IL-17A, and development of severe intestinal inflammation. neutralization of IFNγ in infected *Tslrp KO* mice restored Th2 responses and resulted in worm expulsion (TSLPR indep pathways for Th2 CK production)	mouse	*T. muris*	larvae	healthy	na
Wilson	2011	Induction of colitis. Development of a Th1-dominated response. IL-13 reduces pathology in *Il-10 KO IL-13rα2 KO* mice	mouse	*T. muris*	eggs	*Il-10 KO* spontaneous colitis	na
Vegas-Sánchez	2014	Concurrent infection: **colitis exacerbation, prolonged parasites survival** Colitis at d54 -62 p.i.(all worms expelled): colitis amelioration. Colitis at d27-35 p.i. colitis amelioration, mucosal epithelization and regeneration	mouse	*T. muris*	eggs	DSS colitis	Concomitant Therapeutic
Wilson	2010	**Worsening of colitis**. ↑IFNγ, ↑ IL-17A. ↑IL-13Rα2 resulting in ↓ IL-13 activity	mouse	*T. muris*	eggs	*Il-10 KO* spontaneous colitis	Therapeutic

Author	Year	Effect	Species	Parasite	Component	Disease Model	Timing
			Trichuris (Nematode)				
Broadhurst	2012	Attenuation of colitis. ↑Arachidonic acid metabolism IgE signalling (*Fcer1aA, Ms4a2*), mast cell activation (*Cpa3, Cma1*), Th2 and eosinophil recruitment (*Ccl17, Ccl18, Ccl26*), aaMacs (*Alox5, Alox15*), CK signalling (*Il-5ra, Il9r, Postn*), and worm expulsion. ↓bacterial attachment to the intestinal mucosa and changes in composition of attached bacteria.	macaque monkey	*T. suis*	larvae	Idiopathic chronic diarrhoea	Therapeutic
			Trichinella (Nematode)				
Adisakwattana	2013	Attenuation of colitis. Changes in Treg response.	mouse	*T. papuae*	larvae	DSS colitis	Preventive
Du	2011	Attenuation of colitis. Induction of Th2 and regulatory response; may involve induction of aaMacs.	mouse	*T. spiralis*	53 kDa ES	TNBS colitis	Preventative
Khan	2002	Attenuation of colitis, induction of Th2 response.	mouse	*T. spiralis*	larvae	DNBS colitis	Preventive
Motomura	2009	Attenuation of colitis. Induction of Th2 and regulatory mechanisms	mouse	*T. spiralis*	larval antigens	DNBS colitis	Preventative

aaMacs: alternative activated macrophages, CK: cytokines, CPG: cytosine phosphate guanosine, D/TNBS: Di/Tri-nitrobenzene sulfonic acid, ES: excretory/secretory antigen, DSS: dextran sodium sulphate, IL-13Rα2: IL-13 decoy receptor, in/dep: in/dependent, LPS: lipopolysaccharide, Macs: Macrophages, na: not applicable.

APPENDIX B

Summary of the studies on the therapeutic use of helminth infection or helminth derived products in rodent model of different organic diseases (Figure B. 1).

Figure B. 1: Summary of the studies on the therapeutic use of helminth infection or helminth derived products in rodent model of asthma and allergy, Multiple sclerosis (MS), Reumathoid arthritis (RA), Transplant rejection, Diabetes, Gastritis, Hepatitis

Author	Year	Effect	Species	Parasite	Component	Disease Model
Asthma and allergy						
Herrick	1913	Among the first causal proof linking infection and eosinophilia	guinea pigs	*A. lumbricoides.*	whole body extracts	anaphylaxis
Cho	2015	Suppress airway inflammation, ↑ IL-10, Treg. Via TLR2 on lung EC on TLR2	mouse	*A. simplex*	MIF	OVA-airway allergy
Schnoeller	2008	↓ Th2-related inflammation, OVA-specific and total IgE, IL-4 production. When applied during or after sensitization and before challenge with the allergen. Macs and IL-10 dep.	mouse	*A. vitae*	Cystatin	OVA-airway allergy
Daniłowicz	2013	decreased levels of Th2 cytokines and mast cell degranulation and increased levels of cystatin-specific IL-10	mouse	*A. vitae*	AvCystatin	grass pollen-airway allergy
Rzepecka	2013	Protection associated with restoredTh1/Th2 balance. ↓Th1	mouse	*A. vitae*	ES62	OVA-airway allergy
Rzepecka	2014	Prevent Th2-associated airway inflammation and eosinophil infiltration.↓neutrophil infiltration (therapeutic)	mouse	*A. vitae*	11a, 12b ES62 small molecule analogues	OVA-airway allergy
Bashir	2002	Attenuation of allergy. ↓ PN-specific IgE, f IL-13 by PN-specific T cells.	mouse	*H polygirus*		peanut-allergy model
wilson	2005	↓inflammatory cell infiltrates (lung, CD25 dep). mLN cells: ↑$CD4^+CD25^+Foxp3^+$ T cells, TGF-β expression, and IL-10 responses. mLN (Tregs?)can transfer protection (IL-10 indep)	mouse	*H. polygyrus*	larvae	OVA-airway allergy
Kitagaki	2006	↓eosinophilia, bronchial hyper reactivity, Th2 responses in an IL-10-dependent manner; ↑ IgE	mouse	*H. polygyrus*	larvae	OVA-airway allergy and transfer of OVA-CD4+ T cells
McSorley	2012	At sensitization: ↓esinophilia, ↓ type 2 innate response, ↓Ab generation; ↓ effector T-cell reactivity. ↑$CD4^+Foxp3^+$ Treg At challenge: ↓Eosinophilia	mouse	*H. polygyrus*	E/S antigen	OVA-airway allergy
McSorley	2014	↑ ILC2, ↑IL-5, IL-13 production (via IL-33, MyD88)	mouse	*H. polygyrus*	E/S antigen	Fungal-airway allergy
McSorley	2015	↓ Type 2 CK by ILC2 (at sensitization, MyD88, TRIF indep.)	mouse	*H. polygyrus*	E/S antigen	OVA-airway allergy
Jarrett EE, Orr TS, Riley P.	1971	Parasite infection interferes with albumine passive sensitization for systemic anaphylaxis and skin test reaction	rat	*N. brasiliensis*		Systemic anaphylaxis, skin test reaction
Wohlleben	2004	↓ allergen-induced airway eosinophilia, eotaxin levels. Infection 1-2w before: no effect. ↓albumin-specific IgG1 and IgE (BAL). IL-10 dependent.	mouse, *Il-10 KO*	*N. brasiliensis*	larvae	OVA-airway allergy

Author	Year	Effect	Species	Parasite	Component	Disease Model
Schabussova	2013	↑ IL-10 TGF-β in BMDC (in vitro) .mouse model of birch pollen allergy: ↓allergen-specific antibodies + ↓IgE-dependent basophil degranulation. Prevented airway inflammation	mouse	*O. dentatum*	Whole body extract from male worms (eMOD)	OVA-airway allergy
Mangan	2004	Protection from anaphylaxis. Essential role of IL-10-producing B cells	mouse	*S. mansoni*		systemic fatal anaphylaxis
Smith	2005	↓ CXCL8-induced neutrophil infiltration and inflammation	mouse	*S. mansoni*	smCKBP	Air pouch; contact hypersensitivity
Negrao-Correa	2003	Concomitant infection prolonged airway eosinophilic inflammation (BAL), ↓ viable parasites, ↓ AHR (only during the lung migration phase)	rat	*S. venezuelensis*	larvae	OVA-airway allergy
Rzepecka	2013	Protective, resetTh1/Th2 balance, ↓Th1	mouse	*T. suis*	ES	OVA-airway allergy
Ebner	2014	↓ OVA specific IFN-γ, IL-4 (in vitro). ↓ MHCII and CD86 TLR-9 mediated upregulation by BMDC (in vitro). Mouse: ↓ OVA specific IL-4, IL-5, IL-13 (BAL) and IgE (serum), goblet cells and RELM-α^+ aaMacs, IL-10 mediated. ↑ T. suis specific Th2 response (systemic)	mouse	*T. suis*	ES	OVA-airway allergy
Multiple sclerosis (MS)						
Walsh	2009	Concomitant infection reduced severity. ↓ auto-antigen specific Th1 and Th17 (TGFβ dep). DC induce parasite-specific Treg cells expressing IL-10 and TGFβ.	mouse (WT and IL10 KO)	*Fasciola hepatica*	larvae	EAE
Sewell	2003	Preventive. Th2 shift (STAT6 dep)	mouse (WT and STAT6 KO)	*S. mansoni*	OVA	EAE
La Flamme	2003	Preventive. ↓clinical course, ↓ incidence and delayed onset. ↓ IFN-γ,TNF, IL-12p40 and NO (by splenocytes), ↑IL-10 and TGFβ	mouse	*S. mansoni*	OVA	EAE
Zhu	2012	Concomitant treatment reduced severity, Th2 shift	mouse	*S. mansoni*	LNFPIII	EAE
Reyes	2011	Preventive infection reduced symptoms in 50% of animals, ↓MOG-specific splenocyte proliferation, ↓IL-17, ↓TNF. ↑IL-4 , IL-10, ↓leukocyte infiltration (spinal cord, brain)	mouse	*T crassiceps*	larvae	EAE
Reumathoid arthritis (RA)						
Rocha	2008	Protective. ↓ NO, IL-1β, IL-10	rat	*A. suum*	Extract	ZYA
Rocha	2009	Protective. ↓ IL-10, but not IL-1β or TNF, levels	mouse	*A. suum*	larvae	CIA

Author	Year	Effect	Species	Parasite	Component	Disease Model
McInnes	2003	ES-62 given during collagen priming significantly reduced initiation of inflammatory arthritis. Crucially, ES-62 was also found to suppress collagen-induced arthritis severity and progression when administration was delayed until after clinically evident disease onset	mouse	*A. vitae*	ES-62	CIA
Harnett	2007	PC possess anti-inflammatory activity	mouse	*A. vitae*	ES-62, PC mojety	CIA
Pineda	2012	Target IL-17–producing network, (DC γ/δ or CD4+ T cells signalling). ↓MyD88 and TLRs (by Th17 cells). modulated γ/δ T cells migration (↓CD44), ↓IL-infiltrating cells	mouse	*A. vitae*	ES-62	K/BxN-induced polyarthritis
Al-Riyami	2013	Protective. ↓IFNγ and IL-17	mouse	*A. vitae*	ES62 SMA 11a	CIA
Pineda	2014	Protection, desensitization of the synovial fibroblast responses (IL-22 dep). ↑IL-22 (serum and joints)	mouse	*A. vitae*	ES-62	CIA
Graepel	2013	Worsening. ↑complement C5a and MCP1 (serum)	mouse	*H. diminuta*	larvae	K/BxN-induced polyarthritis
He	2010	Dependent on infection stage	mouse	*S. japonicum*	larvae	CIA
Song	2011	Infection 2w before immunization: reduced CIA severity, ↓ nti-CII IgG, IgG2a, ↑anti-CII IgG1 +; ↓Splenocyte proliferation (vs. polyclonal and Ag-specific stimuli), ↓ IFN-γ, TNF, IL-1β, IL-6 ↑ IL10 by CD4+. Infection at the onset of CIA: ↑Treg, ↓Th17. Infection 1w before immunization: CIA exacerbation.	mouse	*S. japonicum*	larvae (productive infection: ♂+♀ larvae)	CIA
Osada	2009	Infection 2w before immunization: ↓Anti-IIC IgG and IgG2a. ↓IFNγ, TNFα, IL-17A. ↑ IL-4, IL10 up-regulation. Abrogates up-regulation of IL-1β, IL-6 and NFκB (inflamed paws)	mouse	*S. mansoni*	larvae	CIA
Osada	2014	Severity reduced in males, but not in females. Male: ↓ IL-17, TNF reduced, ↑ f IL-4, IL-10 .But: ↑ IgG rheumatoid factor and anti-dsDNA IgG (serum)	mouse (IL-1Ra KO)	*S. mansoni*	larvae	Spontaneous RA
Pearson	1975	Infected mice are protected (incidental finding). After treatment with piperazine: high incidence of adjuvant arthritis	rat	*S. oblevata*	larvae	CIA
Ortiz-Flores	2013	No effect		*T. crassiceps*	larvae	adoptive OVA-specific T cell transfer
Psoriasis						
Atochina	2006	Skin lesions prevention;↓$Gr1^+F4/80^+$ Macs,↓$CD8^+$ T cells	mice [fsn/fsn]	*S. japonicum*	LNFPIII (glycan)	Spontaneous psoriasis-like pathology (flaky skin)

Author	Year	Effect	Species	Parasite	Component	Disease Model
Transplant rejection						
Komine-Aizawa	2011	Reduced resorption rate from 42.9% (control) to 11.1% (rDiAg). ↓ IL-4, IL-23 and TNF (serum)	mouse	*Dirofilaria immitis*	Recombinant polyproteins (rDiAg)	immune mediated pregnancy loss (abortion-prone CBA/J × DBA/2J)
Li	2011	Delayed rejection. ↓$CD4^+$, $CD8^+$, $CD28^+$ T-cells, ↓Fkn mRNA. ↑IL-10 (serum)	rat	*Echinococcus multilocularis*	larvae	orthotopic liver transplantation
ledingham	1996	Delayed rejection. ↓mononuclear cell infiltration (CD4+ and CD8+ T cells). ↑ IL-4 expression (leukocytes)	rat	*N. brasiliensis*	larvae and soluble worm extract	kidney allografts
Liwski	2000	Delayed rejection. ↓cytotoxic T-lymphocyte activity of spleen T cells. ↑IL-4, IL-6 and ↓IFNγ and ↓ proliferation by T cells in response to allo-antigen. ↑$IL\text{-}4^+$ $CD8^+$ Tc2 cells	rat	*N. brasiliensis*	larvae	cardiac allograft
Araujo	1977	Delayed rejection when grafting occurs in the egg-laying phase (60d p.i)	mouse	*S. mansonii*	larvae	skin allograft
GeorgeJ. Svet-Moldavsky	1969	Prolonged allogeneic graft survival.	mouse	*T. spiralis*	larvae	skin allograft
Svet-Moldavsky	1971	Preventive effect 23d before allograft, lost when infection was performed 7d before allograft.	mouse	*T. spiralis*	larvae	skin allograft
Alkarmi	1995	Larvae and larvae secretory antigens delayed allograft rejection. No effect of soluble larval extracts.	mouse	*T.spiralis* or *T.pseudospiralis*	larvae, larvae secretory antigens and soluble larval extracts	skin allograft
Diabetes						
Imai	2001	Insulitis and diabetes prevention. Impaired islet Ag-specific Th1 response. Th2-type shift	mouse (6w old)	*D.immitis*	DiAg	NOD
Lund	2014	Insulitis and diabetes prevention (84% of mice, until 30w). ↓IFN-γ by autoreactive T cell. IgG2a to IgG1switch. M2 switch of peritoneal Macs (Ym1, Arg-1, TGFβ and PD-L1)	mouse (4w old)	*F. hepatica*	Secreted protein	NOD
Liu	2009	Insulitis and diabetes prevention. CD25 and IL-10-independent	mouse (4w old)	*H. polygyrus*	larvae	NOD
Mishra	2013	Infection at 5-7w, inhibited T1D onset (until 40w). ↓ $CD4^+$ T-cell STAT6 phosphorylation. ↑ IL-10 by Tregs. IL-10 dep. Transfer of $CD4^+$ T cells from Hp NOD IL-4 KO mice to NOD mice prevented T1D. IL-10 essential in Th2-deficient environment (not in WT mice).	mouse (IL-4 KO, 5-7 week old)	*H. polygyrus*	larvae	NOD

Author	Year	Effect	Species	Parasite	Component	Disease Model
Osada	2013	Multiple low-dose model: immune mediated 1D: Hp prevented decrease in pancreatic islet size, ↓TNF, IL1β (pancreas, IL-10 and STAT6 indep). Single high-dose: immune independent T1D: no effect of Hp.	mouse (STAT6KO, IFN-γKO, *Il-10 KO*, 6w old)	*H. polygyrus*	larvae	STZ
Hubner	2009	Insulitis and diabetes prevention. Th2 shift in response to main diabetes autoAg: ↑IL-4 and IL-5 αCD3/αCD28-stimulated splenocytes. ↑ insulin-specific IgG1.	mouse (6w old)	*L. sigmodontis*	larvae	NOD
Hubner	2012	Insulitis and diabetes prevention. Despite lack of Th2 shift (NOD.IL4KO). ↑ Treg proliferation (NOD.WT and NOD.IL4 KO). TGFβ dep, Treg indep WT, IL-10R indep (tested with Ab blockade in NOD.WT)	mouse (IL-4 KO)	*L. sigmodontis*	larvae	NOD
Yang	2013	Decreased weight gain and improved glucose metabolism. ↓hepatic steatosis (IL13-STAT6 dep). Preventive and therapeutic	mouse (RIP2-Opa1β cell KO, STAT6 KO, IL-13 KO)	*N. brasiliensis*	larvae	HFD
Ohsugi	1994	↑ disease incidence in a pathogen free environment	mouse (4w old)	*na*		NOD
Cooke	1999	Th2 shift	mouse (4w old)	*S. mansoni*	larvae	NOD
Zaccone	2003	Insulitis and diabetes prevention (<4w age). ↑ IL-10 by T cells, Vα14i NKT cells	mouse (4w old)	*S. mansoni*	soluble extracts	NOD
Zaccone	2009	↑ T regs (pancreas, DC and TGFβ dep).↑ C-type lectins, IL-10 and IL-2. by DCs. ↑TGFβ, Iβ8, Galectins by T cells. Prevention by transfer of spleonocytes from SEA treated mice is dependent on CD25+ T cells.	mouse (6w old)	*S. mansoni*	egg antigen	NOD
Zaccone	2011	↑ FoxP3 (by CD4+ cells), IL-4, ↑ Th2 polarizing DCs. TGFβ and retinoic acid-dep	mouse (5w old)	*S.mansoni*	glycoprotein ω-1 (from egg antigen)	NOD
Zaccone	2010	Induce systemic Th2/Treg response.↑IL-2, IL-6, IL-10, and TGFβ (peritoneal cells), aaMacs (ARG1, FIZZ.1), ↑TGF*β* by DC (in vitro)	mouse (4-6w old)	*Schistosoma mansoni*	egg antigen	NOD
Saunders	2007	Th2 response; prevent β-cell disruption.	mouse (4w old)	*T. spiralis or H. polygyrus*	larvae	NOD
Gastritis						
Martin	2010	Co-infecction transiently reduced gastritis indices. Effect of helminth lost by 42w p.i. ↑ anti-inflammatory mediators mRNA (transient)	gerbils	*B. filariasis*	larvae	*H. pylori* induced gastritis

Author	Year	Effect	Species	Parasite	Component	Disease Model
Whary	2014	Reduced development of gastric atrophy, dysplasia and prevented changes in the gastric flora. ↑ FoxP3+ cells in the corpus.	mouse	*H. polygirus*	larvae	*H. pylori* induced gastritis
Fox	2000	Prevention of corpus gastritis and decreased chronic inflammation. ↓ Th1 CKs	mouse	*H. polygirus*	larvae	*H. felis* induced gastritis
Hepatitis						
Nascimento	2014	↓ liver weight increase and transaminase levels at 8h, 24h an 7d after preventive treatment and at 7d after curative treatment. ↑survival rate from 38.5 %, to 67 % (therapeutic) and to 100 % (preventive). ↓cellular infiltration, ↑IL-4, IL-10, and IL-13. ↑liver fibrosis	Mouse	*A. suum*	Asc extract	Concanavalin A-induced autoimmune hepatitis

aaMacs: alternative activated macrophages, AHR: arways hyper responsiveness, BAL: bronchioalveolar lavage, CIA: collagen induced arthritis, CK: cytokines, CPG: cytosine phosphate guanosine, D/TNBS: Di/Tri-nitrobenzene sulfonic acid, EAE: experimentally induced autoimmune encephalomyelitis, EC: epithelial cells, ES: excretory/secretory antigen, DSS: dextran sodium sulphate, HFD: high fat diet, in/dep: in/dependent, LPS: lipopolysaccharide, na: not applicable, NKT: natural killer cells, NOD: non obese diabetic, OVA: ovalbumin, STZ: streptozotocin

APPENDIX C

Supplementary material for the second manuscript: Administration of *T. suis* Ova protects immunocompetent rabbit from DSS colitis, but is detrimental to immunosuppressed individuals

C.1. Index of Tables

C.2. Index of Figures

C.3. Supplementary Methods

Analysis of peroxidase activity

Peroxidase activity in caecal lysates was measured as previously described[14]. To achieve relative specificity for the neutrophils' MPO the assay was conducted in presence of the EPO inhibitor aminotriazole (3-Amino-1H-1,2,4-triazole, 95%; Brunschwieg, Switzerland) at pH 6.0[296]. MPO activity (indicated as arbitrary units U/g·s) was calculated as mean absorbance (460 nm) per incubation time per protein content of the sample in grams determined by BCA assay (ThermoFisher, Rockford, USA)[297].

Fecal flotation

5 g of fresh feces were collected 1 day before and 1 day after each TSO-gavage and 9 days after the DSS-treatment begin. The samples were immediately suspended in 300 ml water, filtered through a 1mm sieve and incubated at RT for 30 min. The supernatant was discarded and 1 ml of the sediment was resuspended in 10 ml Sheather's solution (454 g Sucrose in 355 ml water with 6ml 37% formalin), mixed briefly and centrifuged 5min at 500 g. 4 drops were collected with a smear loop, transferred to a slide and examined at 10x lens objective, (changing the plane of focus during the examination). As a positive control, 1 ml of the TSO-gavage solution was suspended in 10 ml Sheather's solution and analyzed as described above.

C.4. Supplementary Tables

Table C.1: Scoring system for the daily monitoring of the disease activity index.

Score	*weight loss*	*stool appearance and cecotrophs*	*reduction in food intake*	*reduction in beverage intake*	*fur appearance*
0	None	well-formed solid pellets, 0 cecotrophs	none	none	clean, bright fur
1	0%-2%	easy to smear and loose stool, ≤1 cecotrophs	0%-30%	0%-30%	dim fur
2	2%-5%	loose stool, 2-3 cecotrophs	30%-60%	30%-60%	shagged fur
3	5%-10%	loose smeared stool in cage, 4-5 cecotrophs	60%-90%	60%-90%	smudgy, unclean fur
4	> 10%	loose smeared stool in cage, > 5 cecotrophs	> 90%	> 90%	smudgy, stool-stains, smeared anus

Table C.2: Scoring system for DSS-induced histological changes in the cecum.

	Morphological features			***Inflammation***	
	Villous stunting	**Villous epithelial injury**	**Crypt distortion**	**Intraepithelial lymphocytes**	**LP lymphocytes and plasma cells**
1	Normal mucosa	Normal mucosa	Normal mucosa	5–10/50 IEL/epithelial cells	25% of the villous lamina propria
2	Mild villous stunting	Mild villous epithelial injury	Mild crypt distension, hyperplasia and distortion	11–30 IEL/50 epithelial cells.	25%–50% of the villous lamina propria
3	Moderate villous stunting	Moderate villous epithelial injury	Moderate crypt distension, hyperplasia and distortion.	31–50 IEL/ 50 epithelial cells may be focally clustered.	50%–75% of the villous lamina propria.
4	Marked villous stunting	Marked villous epithelial injury	Marked crypt distension, hyperplasia and distortion	51–100 IEL/ 50 epithelial cells, may be clustered and at all levels of the epithelium	75% – 100% of the villous lamina propria.

Functional annotation was performed usind the David Functional Annotation Tool , 6.7, NIAID/NIH . Genes with FDR<0.1 where included in the analysis. Mouse cecum expression data where obtained from Foth, 2014[230]

Table C.3: detection of *T.suis* specific DNA in intestinal samples of TSO treated rabbits

Treatment	*IS/Ctrl*	*Colitis*	*No*	*Ileum*	*Caecum*	*prox*	*distal*
TSO	**Ctrl**	**DSS**	8.9	-	-	-	-
			8.14	-	-	-	-
			9.4	-	-		
			8.1	-	+	-	-
			8.8	-	+	-	-
			9.9	-	+		
			9.1	-	+		
			9.13	-	+		
			10.7	-	+	-	-
			10.9	-	+	-	-
			10.1	-	+	-	-
			10.15	-	+	+	+
		Water	8.4	-	-	-	-
			8.5	-	-	-	-
			8.12	-	-	-	-
			8.15	-	-	-	-
	IS	**DSS**	7.3	-	-	-	-
			7.2	+	-	-	-
			9.1	-	+		
			9.2	-	+		
			9.14	-	+		
			10.8	-	+	-	-
			9.11	-	+	+	+
			10.3	-	+	+	+
			10.6	-	+	+	+
			10.16	-	+	+	+
		Water	7.1	-	+	+	+
			7.4	-	+	+	+
Veh	**Ctrl**	**DSS**	8.7	na	-	na	na
			8.1	na	-	na	na
			8.11	na	-	na	na
			8.13	na	-	na	na
			9.3	na	-	na	na
			9.5	na	-	na	na
			9.16	na	-	na	na
			10.5	na	-	na	na

			10.12	na	-	na	na
			10.14	na	-	na	na
			8.2	na	-	na	na
		Water	8.3	na	-	na	na
			8.6	na	-	na	na
			8.16	na	-	na	na
			7.5	-	-	-	-
			9.7	na	-	na	na
			9.12	na	-	na	na
		DSS	9.15	na	-	na	na
			10.2	na	-	na	na
	IS		10.4	na	-	na	na
			10.13	na	-	na	na
			9.6	na	-	na	na
			9.8	na	-	na	na
		Water	10.1	na	-	na	na
			10.11	-	-	-	-

Ctrl: control group (immunocompetent), IS: immunosuppressed ; +: positive; - : negative; na: not analyzed.

Table C.4: Genes differentially up-regulated in LPMC and IEC from TSO infected rabbits and in the caecal tissues of T. muris infected mice.

Gene ID	*KEGG pathway*	*Biocarta*	*Gene name*
adam8			a disintegrin and metallopeptidase domain 8
alox15	Arachidonic acid metabolism,		arachidonate 15-lipoxygenase
arg1	Arginine and proline metabolism,	Catabolic Pathways for Arginine , Histidine, Glutamate, Glutamine and Proline,	arginase, liver
arhgdib	Neurotrophin signaling pathway,	Caspase Cascade in Apoptosis, D4-GDI Signaling Pathway, FAS signaling pathway (CD95), HIV-I Nef, TNFR1 Signaling Pathway	Rho, GDP dissociation inhibitor (GDI) beta
arid5a			AT rich interactive domain 5A (MRF1-like)
basp1			brain abundant, membrane attached signal protein 1
c1qb	Complement and coagulation cascades, Prion diseases, Systemic lupus erythematosus,		complement component 1, q subcomponent, C chain
c1qc	Complement and coagulation cascades, Prion diseases, Systemic lupus erythematosus,		complement component 1, q subcomponent, beta polypeptide
ccrl2			chemokine (C-C motif) receptor-like 2
cd14	MAPK signaling pathway, Toll-like receptor signaling pathway, Hematopoietic cell lineage, Regulation of actin cytoskeleton,	Inactivation of Gsk3 by AKT causes accumulation of b-catenin in Alveolar Macrophages,	CD14 antigen
cfp		Alternative Complement Pathway,	complement factor properdin
chi3l1			chitinase 3-like 1
ciita	Antigen processing and presentation, Primary immunodeficiency,		class II transactivator

csf1r	Cytokine-cytokine receptor interaction, Endocytosis, Hematopoietic cell lineage, Pathways in cancer,	CBL mediated ligand-induced downregulation of EGF receptors, METS effect on Macrophage Differentiation,	colony stimulating factor 1 receptor
csf2rb	Cytokine-cytokine receptor interaction, Apoptosis, Jak-STAT signaling pathway,	IL 3 signaling pathway,	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
dhrs9	Retinol metabolism,		dehydrogenase/reductase (SDR family) member 9
fgr	Chemokine signaling pathway,	Roles of αβγ -arrestin-dependent Recruitment of Src Kinases in GPCR Signaling,	Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homolog
gpr141			G protein-coupled receptor 141
hck	Chemokine signaling pathway, Fc gamma R-mediated phagocytosis,	Roles of αβγ-arrestin-dependent Recruitment of Src Kinases in GPCR Signaling,	hemopoietic cell kinase
hdc	Histidine metabolism,		histidine decarboxylase
hk3	Glycolysis / Gluconeogenesis, Fructose and mannose metabolism, Galactose metabolism, Starch and sucrose metabolism, Amino sugar and nucleotide sugar metabolism, Insulin signaling pathway, Type II diabetes mellitus,		hexokinase 3
il1b	MAPK signaling pathway, Cytokine-cytokine receptor interaction, Apoptosis, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, Cytosolic DNA-sensing pathway, Hematopoietic cell lineage, Type I diabetes mellitus, Alzheimer's disease, Prion diseases, Graft-versus-host disease,	Signal transduction through IL1R, IL 5 Signaling Pathway, Msp/Ron Receptor Signaling Pathway, NFkB activation by Nontypeable Hemophilus influenzae,	interleukin 1 beta
il1r2	MAPK signaling pathway, Cytokine-cytokine receptor interaction, Hematopoietic cell lineage,		interleukin 1 receptor, type II
il1rl1			interleukin 1 receptor-like 1

il6	Cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, Cytosolic DNA-sensing pathway, Jak-STAT signaling pathway, Hematopoietic cell lineage, Intestinal immune network for IgA production, Prion diseases, Pathways in cancer, Graft-versus-host disease, Hypertrophic cardiomyopathy (HCM),	Cytokine Network, Erythrocyte Differentiation Pathway, Role of ERBB2 in Signal Transduction and Oncology, IL-10 Anti-inflammatory Signaling Pathway, IL 17 Signaling Pathway, Signal transduction through IL1R, IL 5 Signaling Pathway, IL 6 signaling pathway, Cytokines and Inflammatory Response, Regulation of hematopoiesis by cytokines,	interleukin 6
irf5	Toll-like receptor signaling pathway,		interferon regulatory factor 5
itgb7	Focal adhesion, ECM-receptor interaction, Cell adhesion molecules (CAMs), Intestinal immune network for IgA production, Regulation of actin cytoskeleton, Hypertrophic cardiomyopathy (HCM), Arrhythmogenic right ventricular cardiomyopathy (ARVC), Dilated cardiomyopathy,		integrin beta 7
itk	Chemokine signaling pathway, T cell receptor signaling pathway, Leukocyte transendothelial migration,	The Co-Stimulatory Signal During T-cell Activation,	IL2-inducible T-cell kinase
lcp2	Natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, Fc epsilon RI signaling pathway,		lymphocyte cytosolic protein 2
lpcat2			lysophosphatidylcholine acyltransferase 2
lrrc33			leucine rich repeat containing 33
plek			pleckstrin
ptafr	Calcium signaling pathway, Neuroactive ligand-receptor		platelet-activating factor receptor

	interaction,		
ptpn7	MAPK signaling pathway,		protein tyrosine phosphatase, non-receptor type 7
sell	Cell adhesion molecules (CAMs),	Adhesion Molecules on Lymphocyte, Monocyte and its Surface Molecules, Neutrophil and Its Surface Molecules,	selectin, lymphocyte
serpinb2		Fibrinolysis Pathway,	serine (or cysteine) peptidase inhibitor, clade B, member 2
sh3kbp1	Endocytosis,	CBL mediated ligand-induced downregulation of EGF receptors,	SH3-domain kinase binding protein 1
sla			src-like adaptor
slc45a3			solute carrier family 45, member 3
srgn			serglycin
tgm1			transglutaminase 1, K polypeptide
tnf	MAPK signaling pathway, Cytokine-cytokine receptor interaction, Apoptosis, TGF-beta signaling pathway, TLR signaling pathway, NLR signaling pathway, RIG-I-like receptor signaling pathway, Hematopoietic cell lineage, …	Cadmium induces DNA synthesis and proliferation in macrophages, Cytokine Network, Free Radical Induced Apoptosis, HIV-I Nef, …	tumor necrosis factor

C.5. Supplementary Figures

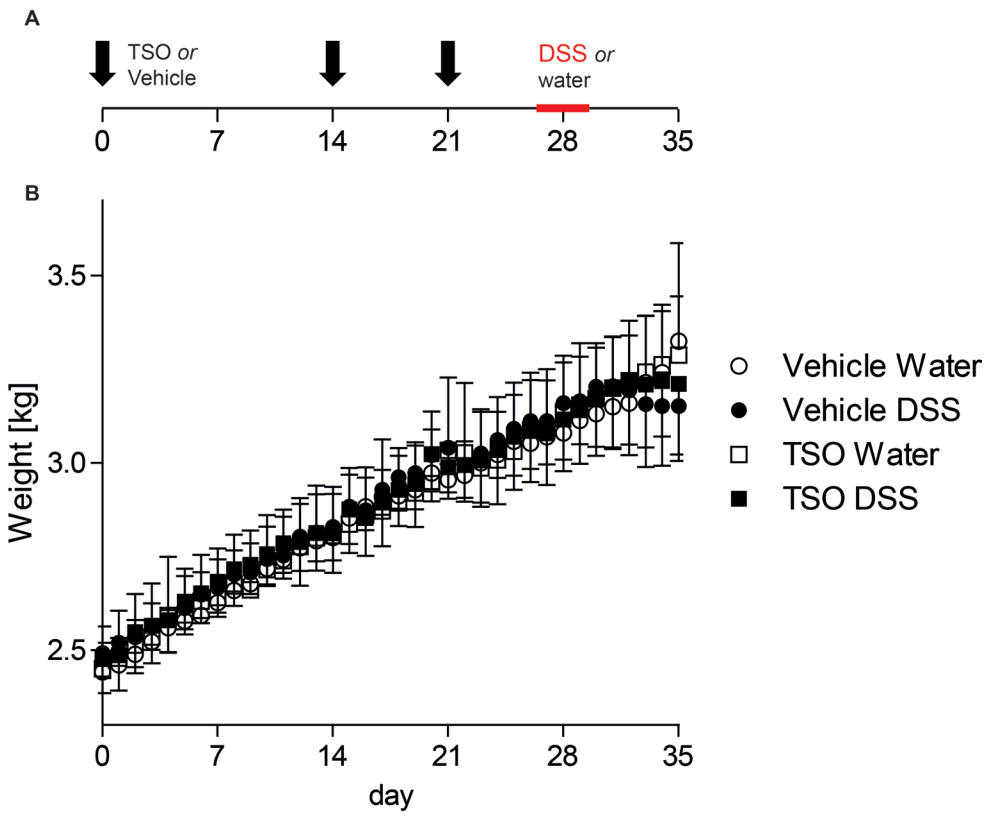

Figure C.1: Preventive treatment with TSO ova in a model of DSS induced acute colitis. A. Experimental layout. B. Weight progression from day 0 (first TSO/Vehicle treatment) to day 35 (day of euthanasia and organ sampling). Colitis was induced at day 26 by administration of 0.1% DSS (w/v) in the daily beverage for 5. Dots represent mean ± SD.

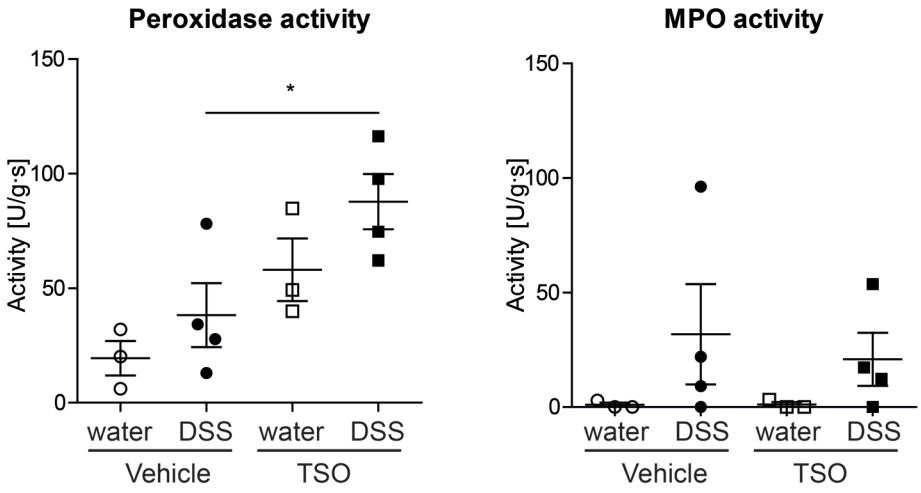

Figure C.2: Neutrophil and eosinophils infiltration into the caecum. Infiltration was determined indirectly by measuring the caecal peroxidase activity. Caecal specimens were excised and homogenized. The supernatants were assayed for the determination of the peroxidase activity with or without the selective eosinophil-peroxidase inhibitor aminotriazole (AMT), activity was normalized for the total protein content as determined by BCA test. Dots represent single animals, bars show mean ± SEM, *P<0.05, unpaired t test.

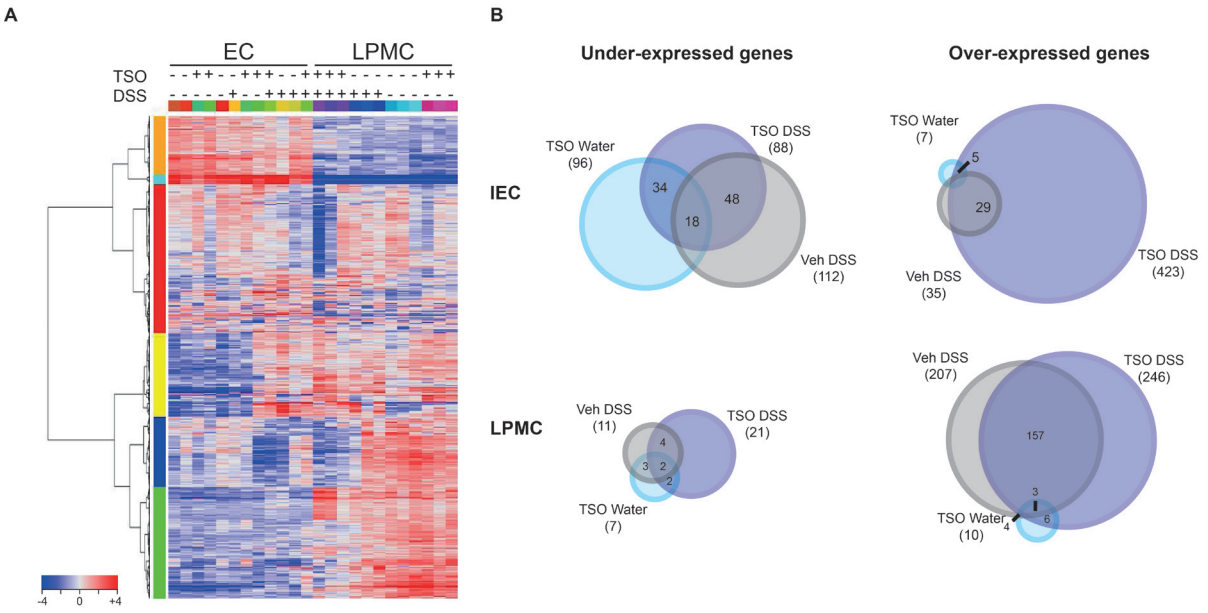

Figure C.3: Genomic signature for genes differentially expressed in LPMC and EC. RNA was prepared from cells isolated from rabbit ceca (n=3 per group) and subjected to genome wide expression analysis. Hierarchical cluster analysis was used to sort expression profiles for the 4 treatment groups. (A) Heatmap of the altered expression of gens in the different groups compared. Each column displays the genomic signature for 1 subject. Genes up regulated are displayed in red, whereas down regulated genes are displayed in blue, as indicated on the accompanying expression scale. (B) Visualization of the under- (left) and over- (right) expressed genes in the TSO DSS, Veh DSS and TSO Water groups in comparison to the control Veh Water group. Genes having a fold change value FC>|1| and P<0.05 were considered differentially expressed genes and were included in the analysis.

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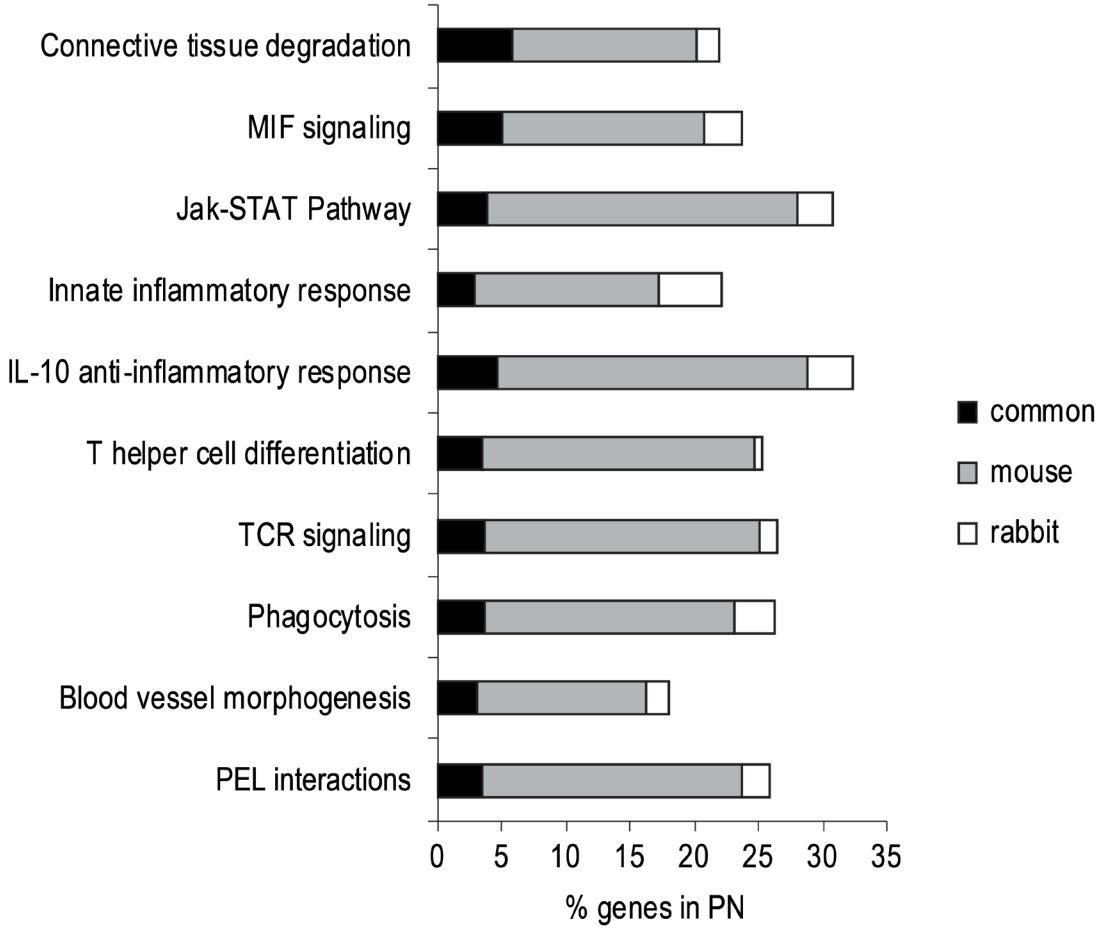

Figure C.4: Process network (PN) analysis of genes differentially regulated in LPMC and IEC from *T. suis* infected rabbits and in the caecal tissues of *T. muris* infected mice. Black bars show the percentage of genes found in both rabbit and mice. The percentage of unique genes is showed in white bars for rabbit and in grey bars for mouse. MIF: Macrophage migration inhibitory factor, PEL: Platelet-endothelium-leucocyte interactions, TCR: T cell receptor. Analysis was performed with metacore. Genes with FDR<0.1 where included in the analysis.

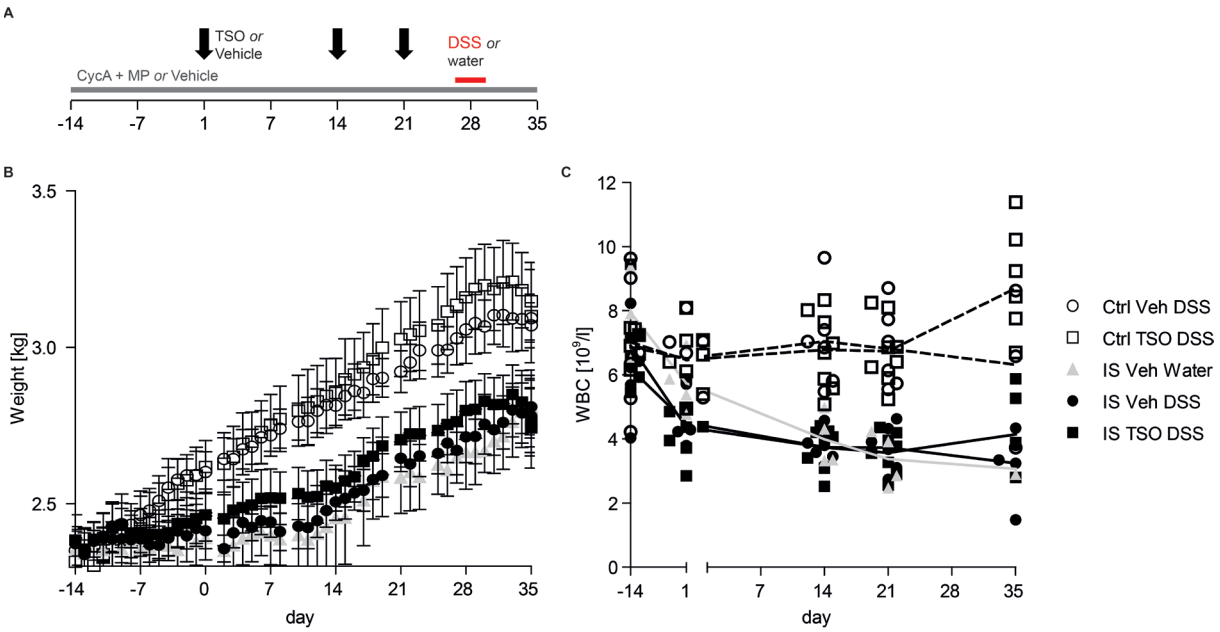

Figure C.5: Preventive treatment with TSO ova in a model of DSS induced acute colitis in immunosuppressed (IS) and immunocompetent (Ctrl) rabbits. A. Experimental layout: IS rabbit received daily Cyclosporine A (CycA) and Methylprednisolone (MP). B. Weight progression from day -14 (start of IS treatment) to day 35 (day of euthanasia and organ sampling) Data show mean± SD from one representative experiment. C. total WBC was measured before the experiment begin (d0) and at day 1, 14, 21 and 35. Dots represent single animals, lines connect arithmetic mean. Data are pooled from 3 independent experiments.

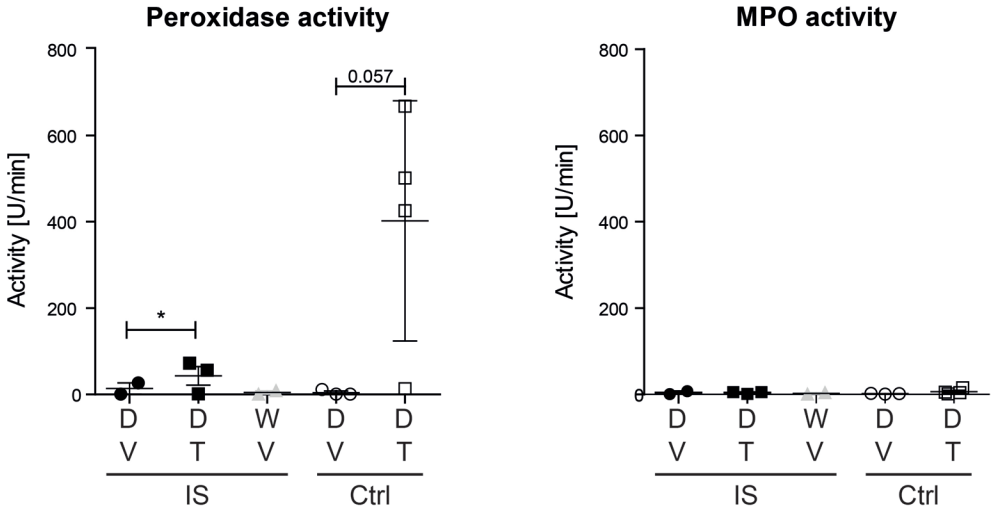

Figure C.6: Neutrophil and eosinophils infiltration in immunosuppressed rabbits. Infiltration into the caecum was determined indirectly by measuring the caecal peroxidase activity. Caecal specimens were excised and homogenized. The supernatants were assayed for peroxidase activity with or without the selective eosinophil-peroxidase inhibitor aminotriazole (AMT); activity was normalized for the total protein content as determined by BCA test. D: DSS, W: water, V: Vehicle, T: TSO, IS: immunosuppressed, Ctrl: control. Dots represent single animals, bars show mean ± SEM, *P<0.05, two-sided pValue, unpaired t test

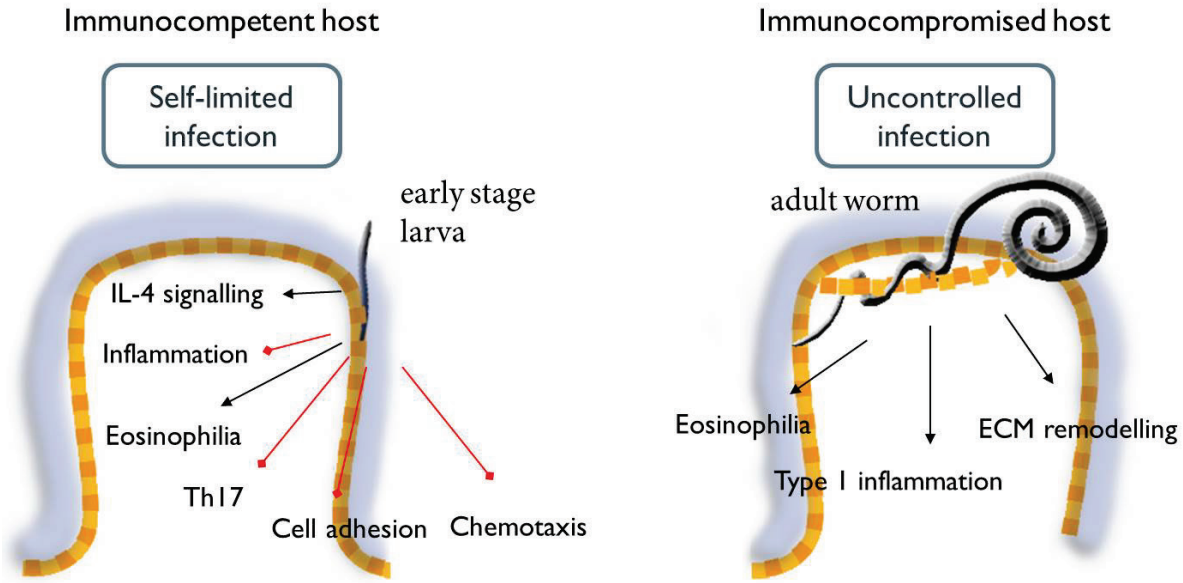

Figure C.7: Modulation of the caecal niche by different stages of *T. suis*. In immunocompetent hosts infection with *T. suis* is cleared before the parasite reaches a late larval stages. *T. suis* larvae exert a beneficial effect on the inflamed mucosa and down-regulates the expression of several colitogenic pathways. In immunocompromised hosts *T. suis* develops further and the protective effect is lost. A strong remodelling of the caecal mucosa occurs and the persistent Trichuris infection leads to an exacerbation of colitis. Black arrows indicate stimulatory pathways; red diamond-tipped arrows indicate inhibitory pathways.